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ASCUS FORMATION AND RECOMBINANT FREQUENCIES IN NEUROSPORA CRASSA

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A NUMBER of years ago it became feasible, as a result of the development of a plating method (LEIN, MITCHELL and HOULAHAN 1948) to obtain prototroph frequencies in samples of several thousand spores from crosses between nutritional mutants. Previously, with a more laborious method, samples of a few hundred spores, or less, had usually formed the basis of linkage information. If linkage is at all close, such small samples give little idea of actual recombinant frequencies. One of the results of application of the plating technique to a study of the order of markers in linkage group IV was the finding of a conspicuous clustering of the markers (MITCHELL and MITCHELL 1954). There appeared to be three groups of rather closely linked markers, these groups being separated by regions of about equal length.

More recently, as a result of examination of other data obtained by the plating method, it was realized that there were, in general, discontinuities in the recombinant frequencies. The recurrence of certain frequencies which gave the clustering effect in group IV was found in other groups as well (MITCHELL 1958). It does not seem realistic to suppose the observed discontinuities to reflect a planned spacing of genes in chromosomes, nor is it obvious that restricted regions of crossing over would be expected to give such similar frequencies in different linkage groups. Moreover, means of testing these two possibilities are not apparent. For these and other reasons which will be discussed, it appears more useful to consider, first, the possibility that in Neurospora it is not the frequency of crossing over that is mainly reflected by the recombinant frequencies.

Recombinant frequencies and ratios of ascus classes

The recurring recombinant frequencies among random spores are indicated in Figure 1. The frequencies are of prototrophs from crosses between nutritional mutants, or of wilds from crosses of two morphological mutants or of a nutritional and a morphological mutant. The sample size was usually between 500 and 8000 spores, the larger samples being, more often, from crosses showing closer linkage. The major portion of the data used has been presented in detail elsewhere (MITCHELL and MITCHELL 1954; MITCHELL 1958). It may be seen that many of the frequencies are concentrated below five percent or around eight, 12 or 24 percent. In linkage group IV frequencies below five percent were usually obtained from crosses between markers within any one of the three clusters mentioned

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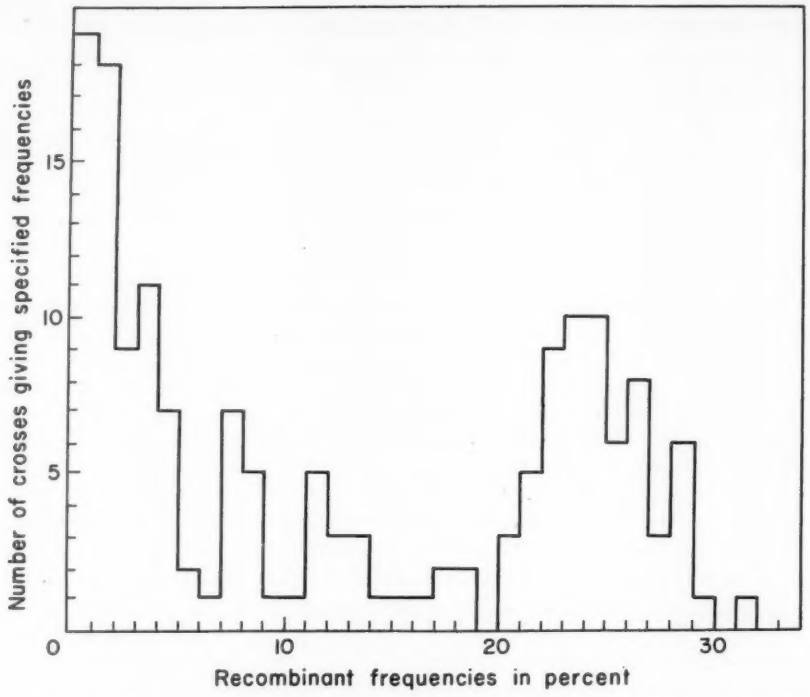

FIGURE 1.—Frequencies of certain recombinant classes among random spores from a variety of crosses. The indicated recurrence of certain frequencies gives the clustered effect of markers in linkage group IV and has been found in other linkage groups also.

above. Crosses involving two of the clusters gave frequencies of about 20 percent, whereas those involving either of these two clusters with the third usually gave around eight or 12 percent.

Two-marker crosses give three general classes of eight-spored asci, parental ditypes, nonparental ditypes and tetratypes (BARRATT, NEWMEYER, PERKINS and GARNJOBST 1954). The first consists of parental segregants only, four of each type, the second of recombinants only, four of each of the two types and the third contains two of each of the four types. If linkage is at all close nonparental ditypes are rare, hence the degree of linkage may often be estimated mainly from the ratio of parental ditypes to tetratypes. For this reason it seems that the discontinuities in recombinant frequencies among random spores might be reflected in ascus data as discontinuities in ratios of parental ditype to tetratype asci. For example, frequencies of 8.3 and 12.5 percent correspond to PD:NPD:T ratios of 2:0:1 and 1:0:1. (PD = parental ditypes; NPD = nonparental ditypes; T =

tetratypes.) An examination of some existing ascus data has revealed indications of preferred ratios of ascus classes from crosses of both linked and unlinked markers. This is illustrated in Table 1 which includes some of the larger samples from two-marker crosses described by HOULAHAN, BEADLE and CALHOUN (1949). The isolation numbers of the mutants and wild types have been used to designate the crosses. In the first seven, the parent shown on the right was a wild type and the second marker scored was mating type, indicated by (*mt*). The samples chosen are of 30 or more asci from *one* cross. (It should, perhaps, be stressed that data pooled from crosses of different isolates or different occurrences of the same mutants, as was done in some cases by BARRATT *et al.* (1954), would not be comparable to the random spore data under discussion. Recurring ratios might well be obscured by pooling data, since, as is well known, crosses involving different isolates of the same mutants may give quite significantly different recombinant frequencies.) Several of the samples, namely 2, 7, 12, 14 and 19, suggest a PD:NPD:T ratio of 1:1:2, while samples 3 and 11 suggest 1:1:4. With respect to the crosses showing linkage, one is, perhaps, mainly impressed by the need for larger samples if preferred ratios are to be recognized. However, there are three crosses (1, 5 and 10) from which the PD:T ratio might be 1:1.

In Table 2 are listed some samples which include no recombinant asci. The relative frequencies of the two classes of parental ditypes shown have been as-

TABLE 1

Observed frequencies of three classes of asci, parental ditypes, nonparental ditypes and tetratypes, from two-marker crosses

	Cross	Number of asci PD	NPD	T	Reference
(1)	15300 *A* × 25*a* (*mt*)*	24	3	27	HOULAHAN, BEADLE and CALHOUN (1949)
(2)	34508 *A* × 25 *a* (*mt*)	7	11	18	"
(3)	34542 *A* × 25 *a* (*mt*)	5	6	22	"
(4)	45304 *A* × 25 *a* (*mt*)	12	17	4	"
(5)	34547 *A* × Ch *a* (*mt*)	19	1	20	"
(6)	37401 *a* × 4 *A* (*mt*)	8	15	20	"
(7)	85902 *A* × Em *a* (*mt*)	11	8	18	"
(8)	263 *a* × 37815 *A*	90	0	8	"
(9)	263 *a* × 67602 *A*	57	0	9	"
(10)	5531 *A* × 37803 *a*	28	1	20	"
(11)	15300 *a* × 34542 *A*	5	7	23	"
(12)	21863 *a* × 34508 *A*	9	12	20	"
(13)	37301 *a* × 37803 *A*	38	0	1	"
(14)	37803 *A* × 39401 *a*	7	9	16	"
(15)	C136 *A* × 51602 *a*	23	31	0	M. MITCHELL, unpublished
(16)	C136 *A* × 70007 *a*	11	8	12	"
(17)	C102 *A* × 37301 *a*	35	0	23	"
(18)	C136 *a* × C102 *A*	14	7	20	"
(19)	C136 *A* × C102 *a*	23	27	59	MITCHELL, 1959

* The symbol, *mt*, indicates that the second marker scored was mating type; the parent on the right is a wild type.

sumed to reflect the frequency of crossing over between the loci of the markers and the centromere of their chromosome, as will be considered below. (PD-1 = "first division segregation" of both markers; PD-2 = second division segregation of both.) Recurring ratios are suggested by the four crosses 2, 5, 8 and 10) from which the PD-1: PD-2 ratio is about 4: 1 or 5: 1.

The author's conclusion is that preferred ratios may regularly occur but have not been noticed because of the scarcity of suitable data. This possibility seems of sufficient interest to merit investigation. Recognition of such ratios may lead to interpretations of genetic behavior which will take into account both the recurring recombinant frequencies and certain observations concerning developing asci which will be discussed below.

Second division segregation and the frequencies of ascus classes

Formation of a tetratype ascus requires that at least one of the segregating markers must appear in each half of the ascus, as follows:

$$a\,b, a\,b, a\,b^+, a\,b^+, a^+b, a^+b, a^+b^+, a^+b^+$$

Or, as this is often stated, it must segregate at the second division. Thus, the frequency of tetratype asci depends upon the frequency of segregation of markers at the second division.

It has been assumed that the four spore pairs of an ascus represent the products of meiotic division of a single diploid zygote nucleus called the primary ascus nucleus. The centromeres are assumed to segregate regularly at the first division; segregation, as in the ascus above, of the *b* and b^+ factors at the second division is assumed to be accomplished through crossing over between the *b* locus and the centromere of its chromosome. Thus, it is said that the frequency of segregation of a marker at the second division gives a measure of the distance from the centromere to the locus in question.

TABLE 2

Observed frequencies of two classes of parental ditypes from two marker crosses which gave, in the samples considered, no recombinant asci

	Cross	Number of asci† PD-1	PD-2	Reference
(1)	55701 *a* × 1 *A* (*mt*)*	36	2	HOULAHAN, BEADLE and CALHOUN
(2)	263 *A* × 44204 *a*	24	6	"
(3)	15300 *A* × 34508 *a*	19	13	"
(4)	27663 *a* × 44206 *A*	55	3	"
(5)	27663 *a* × 70004 *A*	28	5	"
(6)	35405 *A* × 37803 *a*	42	5	"
(7)	37301 *A* × 37815 *a*	18	18	"
(8)	37301 *a* × 37815 *A*	33	7	"
(9)	37301 *a* × 67602 *A*	20	9	"
(10)	37815 *a* × 67602 *A*	26	5	"

* See Table 1.
† PD-1 = "first division segregation" of both markers; PD-2 = "second division segregation" of both.

Because of its simplicity this is undeniably an attractive hypothesis but, to the working geneticist, it has not been a very useful one. The second division segregation frequency of a given marker may vary in different crosses from almost zero to around 50 percent and variation appears to be more the rule than the exception. Hence, obtaining a value for the "centromere distance" of all markers in a linkage group may give little information as to which markers will show close linkage when crossed together.

A further objection to the crossing over hypothesis arises from assumptions which it is necessary to make regarding orientation of the meiotic spindles. If, as it has been supposed, heterogeneity of each of the two halves of an ascus with respect to a marker and its normal counterpart demonstrates segregation of the determinants concerned at the second meiotic division, then it must be assumed that the meiotic spindles are parallel to the long axis of the ascus and that the second division spindles do not overlap. The author has been able to find little or no justification for these assumptions by observing stained preparations of developing asci. Few spindles were seen and, when seen, appeared to be following no rule regarding their orientation. Some appeared to be longitudinal but others appeared transverse, oblique or even spiralled. Overlapping spindles were also seen, sometimes parallel, and sometimes almost at right angles to one another.

It is now the author's opinion that the "second division segregation" frequency is more a property of the cross than of the individual markers and that the mechanism which determines it, and, through it, the tetratype ascus frequency, is best regarded as unknown.

Nuclear divisions in asci

The more familiar interpretation of events in developing asci is given by McClintock (1945) and Singleton (1953). According to this interpretation the ascus arises from a single cell which contains two haploid, nonsister nuclei. The diploid primary ascus nucleus, referred to above, arises by fusion of these two haploid nuclei. Fusion is immediately followed by meiosis to give four haploid nuclei which then divide mitotically so that the octanucleate ascus consists of four pairs of sister nuclei contained in a common cytoplasm. Spores are then cut out around each one of the eight nuclei which divide again mitotically to make the spores binucleate.

When the author first became interested in examining developing asci, the question in mind was not whether meiosis occurs in the ascus but whether irregular chromosome distribution occurred with a significant frequency. Observations made over a two-year period and on a rather wide variety of crosses have, however, led to the conclusion that meiosis does not regularly occur in the ascus, at least not at a time when the ascus is recognizable as such, and that the figures which suggest meiotic divisions in recognizable asci largely represent exceptional events, or events misplaced in time. This conclusion is based on a number of details, many of them minor in themselves, but, taken together, have led the author to reject the picture of ascus development outlined above. Some of these details will be described as examples.

The methods used are not known to differ effectively from those of SINGLETON (1953) except, perhaps, in three particulars, namely that phase contrast microscopy was used throughout; the preparations were squashed in some cases, but not routinely; the staining was usually light.

The first detail to raise doubt is one which SINGLETON had reported (without comment) but of which the author was unaware. This is that the enormous growth of the ascus (at least a tenfold increase in size) must, in the conventional scheme, be assumed to take place mainly between the time of fusion of the two haploid nuclei and the first anaphase of meiosis. Yet no resting stage of the nucleus during this period has been described nor has any indication of one been seen by the author. In asci of intermediate sizes the large nucleus appears regularly to be in pachytene of prophase I, whereas asci showing diakinesis, metaphase I and anaphase I figures are regularly of about maximum size. To one accustomed to the rule that a uninucleate cell does not grow while its nucleus is undergoing division, this is disturbing. It seems unlikely that the ascus merely swells by taking up water during the period of apparent growth and then fills in its cytoplasm later when the meiotic divisions are completed. One would not expect differentiation of the ascus wall to accompany swelling, whereas, in fact, the curious structures in the apical pore region are regularly seen in asci showing metaphase I and anaphase I figures but not in those with the pachytene nuclei. Also, the long, narrow shape of the fully grown ascus seems an unlikely one to be achieved by swelling. The "primary ascogenous cell" is roughly triangular in shape and the young ascus, said to grow out from it, has much the shape of an elongated drop. Formation of a long, slender cylinder by swelling of either of these seems improbable.

As SINGLETON has pointed out, the various nuclear stages tend, rather generally, to be associated with certain characteristics of ascus morphology. This, as he suggests, is convenient for the cytologist but it is also sometimes puzzling. In some cases the morphological traits are such as to make it appear unlikely that asci showing a particular nuclear stage could develop from those showing stages just preceding. For example, in several crosses it was noticed that late anaphase III asci were, quite regularly, both longer and wider than those in which eight prophase nuclei could be seen. Thus, if these two types of asci represented successive stages of development, considerable shrinkage would have had to accompany the transition from anaphase III to prophase IV. From some other crosses the asci appeared more uniformly slender and among them ones with four "prometaphase III" nuclei were seen with a low but fairly regular frequency. Again, a difference was regularly seen between tetra- and octanucleate asci in that the contents of the former seemed to be distributed un-uniformly, so as to suggest less dense areas in the "cytoplasm," usually more towards the ends of the ascus. From these same crosses full-length asci with four loosely spaced spores were seen with a frequency corresponding roughly to that of the tetranucleate asci. This suggests the possibility that the more mature condition of a tetranucleate ascus was four spored rather than octanucleate or eight spored. Another example is offered by the situation with respect to diplotene nuclei, which SINGLETON has described as "refractory

subjects for detailed observation." The author, however, cannot claim even to have observed transitions from pachytene to diakinesis. The larger asci showing the familiar pachytene nucleus with a very large nucleolus appear, rather regularly, to be degenerating, with the nucleolus weakly stained and the chromatin threads fuzzy in appearance. Diakinesis figures observed were like those shown in SINGLETON's illustrations, with contracted chromosomes and much smaller, often irregularly shaped, but well-stained nucleoli.

Another feature, disturbing to the author, is the infrequence with which nuclei showing contracted chromosomes are to be seen in asci. This, taken at its face value and coupled with other details such as the failure to find certain transition stages, is thought to suggest that the nuclear division figures observed in asci represent exceptional events rather than successive stages in a single, regularly occurring, developmental process.

Structural complexity of the ascus

A substitute scheme tracing the derivation of the ascospore nuclei cannot be given at this time. It can be said, however, that an ascus which is recognizable as such does not appear to represent a single cell which becomes divided into eight spores. Rather, it seems to consist of an outer wall within which several individual hyphae are coiled intimately with one another. The spores and their matrices appear to be formed from these inner hyphae. This can be seen rather readily, at least with phase contrast, in preparations of asci which are not degenerate and which have not been squashed. The inner walls appear to be fragile and to be easily collapsed by pressure so that, with squashing, one may obtain the appearance of the "common mass of cytoplasm" referred to by SINGLETON, even in preparations containing many "healthy" asci. In degenerate, much vacuolated asci, (such as those shown in many of SINGLETON's figures) one might expect structural details often to be lost, particularly after squashing.

The sketches shown in Figure 2 indicate that the ascus content is intricately coiled and suggest that the coiling is related to spore formation but give little indication that several hyphae are involved in the coils. The asci sketched are thought to have been too roughly handled to show this. Also, any or all of them may have been degenerating since mature fruiting bodies of the cross from which they came contained a large proportion of degenerate asci. There is, however, good reason for stating that there are several inner hyphae per ascus. On more carefully prepared slides it has been possible in some cases to see, at almost any point along the length of the ascus, at least two and possibly three or more individual strands. Also, differences between the strands with respect to certain morphological details can sometimes be seen.

The fact that the inner hyphae appear to be very thin walled and can sometimes be seen to have septa has directed attention to the masses of hyphae which regularly surround the asci. These hyphae are thin walled and septate and often appear to be of two types. Type A, except for the thinness of its walls, resembles vegetative hyphae in appearing like a tube interrupted by fairly regularly spaced cross walls or septa. Type B hyphae appear larger in diameter and, because they

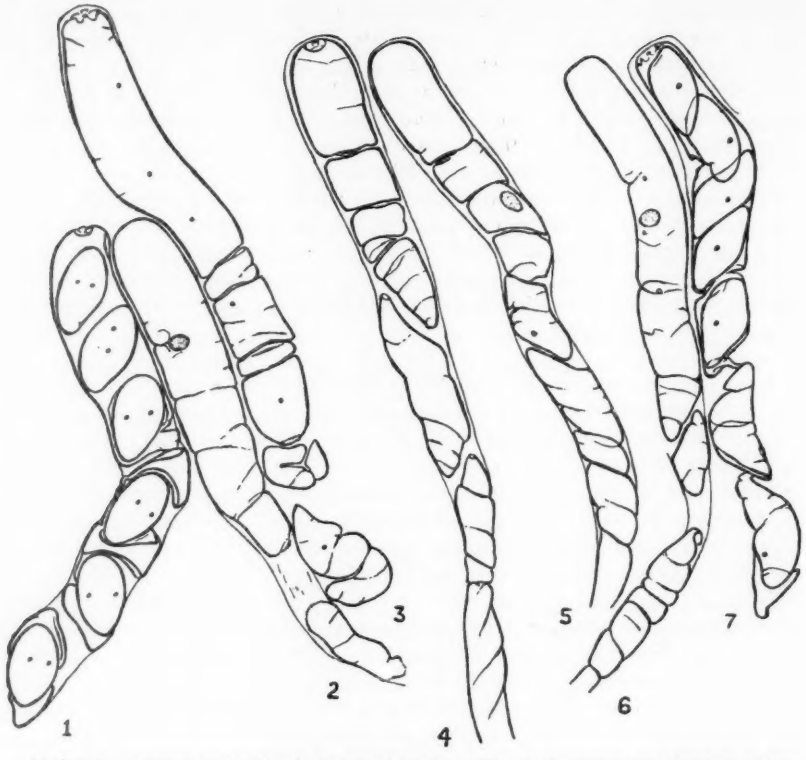

FIGURE 2.—Camera lucida sketch, at magnification ×800, of immature asci thought to have been stretched in preparation of the slide. The outer walls of asci 3, 6 and 7 were obviously broken. Attention is called to the coiled and segmented appearance of the ascus contents. This is often seen even when, as in asci 2, 5 and 6, the large "prophase I" nucleolus is apparent. Visible presence of the large nucleolus usually coincides with lack of visible structure in the apical pore region, however. Some indication of the details of coiling involved in spore formation may be seen in ascus 7. The proximal segment of ascus 3 is thought to represent one spore with its accompanying matrix as seen around the proximal spore of ascus 1. The asci were all obtained from the same cross but the two groups, 1, 2 and 3 and 4, 5, 6 and 7 came from different perithecia.

are constricted at each septum, resemble more chains of "cells" which are roughly the size and shape of ascospores. These hyphae presumably correspond to the sterile hyphae referred to by SINGLETON. However, the croziers, or ascus-forming hyphae of the conventional scheme, can be seen to arise from them.

The reluctance to speculate about derivation of the ascospore nuclei stems in part from observations, not accounted for by the conventional scheme, concerning nuclei of "sterile" and ascogenous hyphae. "Cells" of the "sterile" hyphae usually appear to contain around four to eight small nuclei each. Nuclei of the

croziers, on the other hand, are usually much larger and, as SINGLETON has mentioned, resemble the ascospore nuclei in size and appearance. In the conventional scheme nuclei in these three locations are said to be "haploid" yet no explanation has, to the author's knowledge, been given of the rather impressive difference in size between the "haploid" nuclei of the "sterile" hyphae and those of the croziers and ascospores. Because ascus-forming hyphae can be seen to arise from "sterile" hyphae it seems pertinent to ask what lies back of so marked a difference in the nuclei. One obvious possibility which suggests itself is association of small nuclei to form larger ones. This recalls an alternative scheme developed in connection with related fungi by GWINNE-VAUGHAN and BARNES (1927), HARPER (1905) and others. According to this scheme, nuclear fusions precede the formation of ascogenous hyphae so that their nuclei are diploid rather than haploid; subsequent fusion in the young ascus forms a tetraploid primary ascus nucleus which undergoes double reduction to give haploid ascospore nuclei. Possibly one of the details considered in formulating this scheme was the difference in size of nuclei of "sterile" and ascogenous hyphae. In any case it serves to illustrate the fact that cytologists have not been in agreement with regard to the derivation of ascospore nuclei.

Variations in size, morphology and ability to mature of asci from any one cross are among the stumbling blocks thought to impede the author's attempt to form a coherent picture of ascus development. However, crosses within inbred lines are being studied with the hope of separating variations in the pattern of development and thus decreasing the complexity of the intertwined mass of hyphae within which the early stages of ascus development appear to take place. Thus, by working backward from spore-containing asci, it may be possible to follow the associations of hyphae which are thought to accomplish the formation of asci with spores.

Ambiguities with respect to the site of determination of ascus phenotypes

If diploidization and meiosis take place in the ascus, as in the scheme outlined by SINGLETON, then, with respect to the determination of segregation patterns, each ascus represents a separate event. This appears to be contradicted, however, by the regular occurrence of identical twins among asci of many *N. crassa* crosses (and of the one strain of *N. tetrasperma* examined). The two members of such a pair of asci are adjacent in the ascus cluster and appear identical in size and in other morphological details as well as in degree of maturity or degeneration. Although a few preliminary results (MITCHELL 1959) suggest that twin asci may regularly be alike with respect to segregation patterns of familiar markers, little is known about this at present. However, the fact that degenerate twins regularly occur, both showing the same symptoms of degeneration, makes it appear likely that the phenotype with respect to degeneracy is determined simultaneously in both members by the same events. This is consistent with their identical age and close association in the ascus cluster. Therefore, even in the absence of more extensive genetic data it is, perhaps, worthwhile to stress the irreconcilability of this situation with the conventional scheme of ascus development as it now stands.

Other observations concerning the ability to produce normal ascospores suggest segregation of the determining factors in ascus-forming hyphae. As is well-known to those who dissect asci of *Neurospora crassa*, mature fruiting bodies of the majority of crosses contain large numbers of asci which have failed to mature and which, in many cases, appear degenerate. This is often dismissed on the supposition that required nutrients were exhausted before maturation of all asci could take place. Dissatisfaction with this explanation has been created by the observation that immature fruiting bodies of such crosses often appear to contain about the same proportions of normal- and degenerate-appearing asci as do the mature ones. Although there is considerable variation, asci seen in many mature fruiting bodies may be roughly classed as follows: degenerate-appearing asci in which no spores are seen; asci containing only degenerate-appearing spores; asci containing only normal-appearing spores; asci in which both normal- and degenerate-appearing spores are seen. If segregation and recombination of degeneracy-determining factors occur only in the ascus, then one might expect the frequency of the last class often to approach, at least, that of one or more of the first three classes. The fact that the last class is more usually found to be far outnumbered by the other three is thought to suggest, that, with respect to degeneracy, the phenotype of the ascus is at least partly determined prior to its formation, during the production of the ascus-forming hyphae.

The extent to which the ascus phenotype is predetermined in ascogenous hyphae must be judged on the basis of future data. However, it already appears useful to consider other mechanisms of recombination and segregation since, in doing so, it may be possible to take into account the various inconsistencies in ascospore development schemes and to use the recurring recombinant frequencies instead of dismissing them as just another mystic attribute of the (in Neurospora) hypothetical process of crossing over.

SUMMARY

Observations are considered which are thought to conflict with, or to be unaccounted for by the more familiar interpretations of nuclear events in the sexual cycle of *Neurospora crassa*.

1. Preferred recombinant frequencies and, possibly, preferred ratios of certain segregation classes among asci, are found but are not predicted by interpretations which relate these frequencies directly to frequencies of crossing over.
2. Statements concerning orientation of meiotic spindles, made in support of the crossing over hypothesis designed to account for the occurrence of tetratype asci, appear unjustified by observable details.
3. Details observed in connection with the rather infrequent nuclear division figures seen in asci suggest that such figures largely represent exceptional events rather than nuclear conditions associated with successive steps in a single, regularly occurring pattern of ascospore formation.
4. The ascus itself appears to represent a complex structure formed by associations of several hyphae which cooperate in forming its spores, rather than a single, uninucleate cell which grows and divides to form spores.

5. The regular observation of pairs of adjacent asci which appear to be identical twins conflicts with the assumption that the determination of phenotype in each ascus represents an independent event. Observations concerning frequencies of aborted and partially aborted asci suggest that the phenotype of the ascus may be, in part, determined prior to its formation, in the hyphae from which it is formed.

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LITERATURE CITED

BARRATT, R. W., D. NEWMEYER, D. D. PERKINS, and L. GARNJOBST, 1954 Map construction in *Neurospora crassa*. Advances in Genet. **6**: 1–93.

GWYNNE-VAUGHAN, H. C. I., and B. BARNES, 1927 *The Structure and Development of the Fungi*. Cambridge.

HARPER, R. A., 1905 Sexual reproduction and the organization of the nucleus in certain mildews. Carnegie Inst. Wash. Publ. **37.**

HOULAHAN, M. B., G. W. BEADLE, and H. G. CALHOUN, 1949 Linkage studies with biochemical mutants of *Neurospora crassa*. Genetics **34**: 493–507.

LEIN, J., H. K. MITCHELL, and M. B. HOULAHAN, 1948 A method for selection of biochemical mutants of Neurospora. Proc. Natl. Acad. Sci. U.S. **34**: 435–442.

MCCLINTOCK, B., 1945 Neurospora. I. Preliminary observations of the chromosomes of *Neurospora crassa*. Am. J. Botany **32**: 671–678.

MITCHELL, M. B., 1958 Genetic recombination in Neurospora. Genetics **43**: 799–813.
1959 Detailed analysis of a Neurospora cross. Genetics **44**: 847–856.

MITCHELL, M. B., and H. K. MITCHELL, 1954 A partial map of linkage group D in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **40**: 436–440.

SINGLETON, J. R., 1953 Chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa*. Am. J. Botany **40**: 124–144.

VARIEGATION IN THE ZESTE EYE COLOR ALLELES AND ITS BEARING ON GENE ACTION DURING THE DEVELOPMENT OF THE EYE OF DROSOPHILA MELANOGASTER[1]

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TWO of the four available zeste alleles show a variegation pattern of large red spots on a lighter colored background. The shape of these spots is identical to the shape of X-ray-induced mosaic spots that have been used earlier in an analysis of cell lineage and developmental physiology of the eye anlage (BECKER 1957a, b). In the present paper this identity will be used to analyse the variegation pattern of zeste eyes. This pattern will also be compared with the similar one of the eye deformations in Lobe alleles (see also BECKER 1957a, b). The data on the zeste alleles will then be used to add to the present knowledge of eye development. Finally, the particular type of gene action encountered in both the zeste and Lobe alleles will be discussed. The salt-and-pepper mottling that is also displayed by flies with the zeste alleles will be described but will not be analysed any further.

MATERIAL AND METHODS

The zeste locus lies in the X chromosome at 1.0. The two alleles z (zeste) and z^a (zeste-a) were found by M. GANS, the allele z^m (zeste-mottled) by M. M. GREEN. Stocks carrying these three alleles—z, $y\ z^a$ and $sc\ z^m$ (y, yellow; sc, scute)—were kindly provided by DR. M. M. GREEN. The fourth allele arose spontaneously in one male of the $sc\ z^m$ stock during the investigation. It was designated z^l (zeste-light), the stock accordingly $sc\ z^l$. Salivary gland chromosomes from a stock carrying z (GANS 1953) and from a stock carrying z^m were found to be normal.

Temperatures at which cultures were kept during the experiments will be given at appropriate places.

In X-ray experiments larvae of all stages were uniformly given the dose of 1250r. Two different machines had to be used; the data for one were: 100 kv, 6 ma, no filter, 200r/min, and for the other one were: 250 kv, 15 ma, 0.5 mm Al filter, 120r/min. This difference is, however, believed to be unimportant in these experiments.

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RESULTS

The zeste alleles

A thorough description and genetic analysis of the allele *z* has been given by M. GANS (1953). The pigmentation of the eyes is different in both sexes, and it also depends on the temperature under which the flies are raised. The characteristics of *z* and of the other three alleles are assembled in Figure 1. Cultures of stocks with all four alleles were raised at 14, 17, 25 and 30°C. Since the 14°C cultures showed the same features as the 17°C cultures, only to a slightly more extreme degree, they are summarized in the table as one group, "low". The same is true for the 30 and 25°C cultures respectively; they are summarized in group "high".

z: Females raised at high temperatures have lemon-colored eyes. When raised at low temperatures they show a salt-and-pepper type of variegation consisting of fine red spots on a lemon background. At 17°C there is a region of darker, more densely mottled ommatidia along the posterior margin of the eye. At 14°C almost the whole eye has this darker appearance, and only a narrow region at the anterior margin has the looser, lighter kind of mottling. In Figure 1 the appearance of an eye developed at 17°C is given. At first glance the eyes of males seem to be normal in cultures kept at all different temperatures. Closer inspection, however, especially under mineral oil, shows that in the eyes of males raised at high temperatures the pigmentation is incomplete. In these males the hexagonal pattern of red pigment—hexagonal because of the location of pigment cells on the borders between ommatidia—is interrupted at various places in the eye.

z^m: Females raised at high temperatures have lemon-colored eyes. When raised at low temperatures they are, especially at 14°C, slightly orange. They do not show any salt-and-pepper mottling, but have large consolidated spots instead. These spots are normal red or darker. The occurrence of these spots in any type of fly is represented in Figure 1 by one single, but typical, spot of this kind. Males brought up at high temperatures show the same type of large spots on eyes that

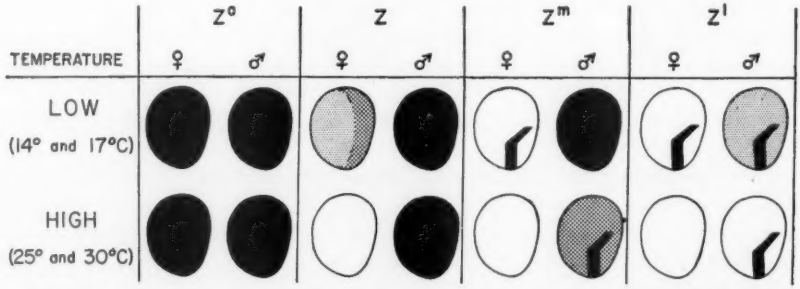

FIGURE 1.—Schematic representation of left eyes of the zeste alleles. Black = normal red; dotted = salt-and-pepper mottling; white = lemon; the black spot in z^m and z^l represents a c.l. spot.

otherwise have a lemon background with salt-and-pepper mottling. The eyes of low-temperature males have the same (almost normal) pigmentation as z males at high temperatures.

z^l: The eyes of these females are lemon color at all temperatures and only at 14°C show spots of the large type. Males raised at high temperatures show the characteristics of large spots most clearly, since the background color of the eyes is uniformly lemon without any other mottling. At low temperatures they look similar to z^m males raised at high temperatures, i.e., large red spots on eyes that otherwise have a lemon background with salt-and-pepper mottling.

z^a: This is a normal isoallele of the zeste locus, i.e., the eyes of both sexes are normal under all temperature conditions, and the zeste character shows only when z^a is in compound with any of the other three alleles.

The conclusions reached from the description of the four alleles are: In Figure 1 the four alleles are arranged according to increasing lightness from left to right. There is a consistent order from darker to lighter phenotypes from the upper to the lower part of the figure, i.e., as the culture temperature is increased. Males carrying any allele raised at a high temperature have a phenotype very similar to males of the next lighter allele raised at a low temperature. The difference in pigmentation due to temperature is not only a matter of the amount of pigment, but also, as was described in the z females, of its distribution over the eye. The pigment that caused the darker, more densely mottled area covers a greater area of the eye when the flies were raised at 14°C, compared to 17°C. The increase in pigmentation with decreasing temperatures proceeds obviously in a posterior-anterior direction. Two of the four alleles show, under appropriate temperature conditions, large uniformly red pigmented areas on a lemon background or on a lemon background with salt-and-pepper mottling.

The compounds

The eyes of compound females will give some further information about the characteristics of the alleles, especially about z^a.

The eyes of all flies raised at high temperatures are lemon. At low temperatures they show the following features: Whenever one of the alleles is z^a or z, the eyes show a salt-and-pepper mottling (the first five compounds in Figure 2). Whenever one of the alleles is z^m or z^l, the eyes show large uniformly pigmented spots typical for these two alleles (the last five compounds in Figure 2). Either these spots appear in addition to the salt-and-pepper mottling or they are the only type of variegation, as in z^m/z^l.

The indicated order of the alleles (as previously made) according to the darkness of pigmentation ($z^a \rightarrow z \rightarrow z^m \rightarrow z^l$) also holds true in their compounds. z^a/z is the darkest; z^m/z^l is the lightest. Among the z^a-containing compounds the order from dark to light is $z^a/z \rightarrow z^a/z^m \rightarrow z^a/z^l$. The darkness is obviously due to the distribution of pigment in the eye, which varies, as in z/z females, along a posterior-anterior gradient. z^a/z flies raised at 17°C show a pigment distribution similar to that described for z females raised at 14°C, i.e., most of the eye is densely mottled and dark, with only a small part along the anterior margin somewhat lighter. On

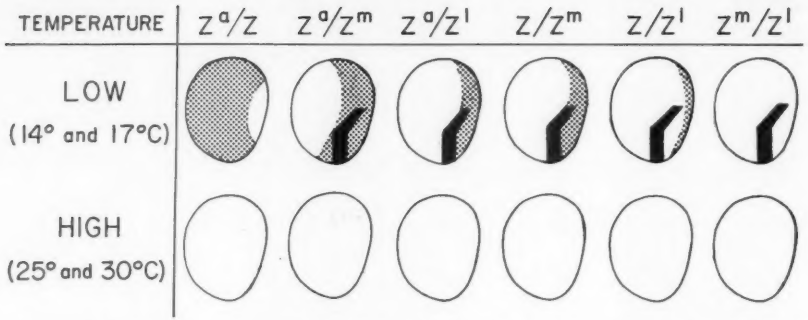

FIGURE 2.—Schematic representation of the left eyes of the zeste compounds. For further details see Figure 1.

the other hand, when z^a/z flies were raised at 14°C, the pigment-forming factor appears to have travelled anteriorly across the eye, leaving the posterior rim without red pigment: the eye is darkly mottled except for a small posterior middle portion. This condition is represented in Figure 2.

The compounds reveal one other property of the allele z^a; that is with regard to the formation of the large uniformly pigmented spots. The compound z^a/z does not show this type of spot. In compounds of either one of these two alleles with z^m or z^l this spot type is found. It seems, therefore, that z^a is, like z, an allele that does not allow on its own the formation of large spots.

The origin of the large spot variegation in z^m *and* z^l

Figure 3 gives an idea of the variety of large spots that are formed in z^m and z^l. The pictures have been drawn from z^m males raised at 25°C. Figure 3 could also represent the spotting in z^m and z^l females raised at low temperatures. Because of their low spot incidence, however, eyes with large dark areas like those in Figure 3a will very seldom be found among these females. This matter will be discussed later in more detail.

As was mentioned earlier, these spots are identical in shape to mosaic spots, which can be produced by the irradiation of w/w^{co} larvae (w, white; w^{co}, white-coral). In that case, by X-ray induction of somatic crossing over in one presumptive eye cell, both its daughter cells were marked genetically; one of two sister cells became homozygous w/w, the other one w^{co}/w^{co}. Both of these cells keep dividing along with the rest of the eye anlage and finally yield a white and an adjacent white-coral spot in the otherwise intermediately colored w/w^{co} eye (see Figure 1 in BECKER 1957a). The size of these spots depends on the stage of the larvae at the time of irradiation; they are large when younger and small when older stages are irradiated. Figure 4 shows four examples of these "twin spots". In this case embryos and larvae with the constitution *sc* z^m w^+/sc^+ z^+ w were X-rayed. Crossing over between the white locus and the kinetochore in the X chromosomes of an eye anlage cell yields a twin spot consisting of one lemon

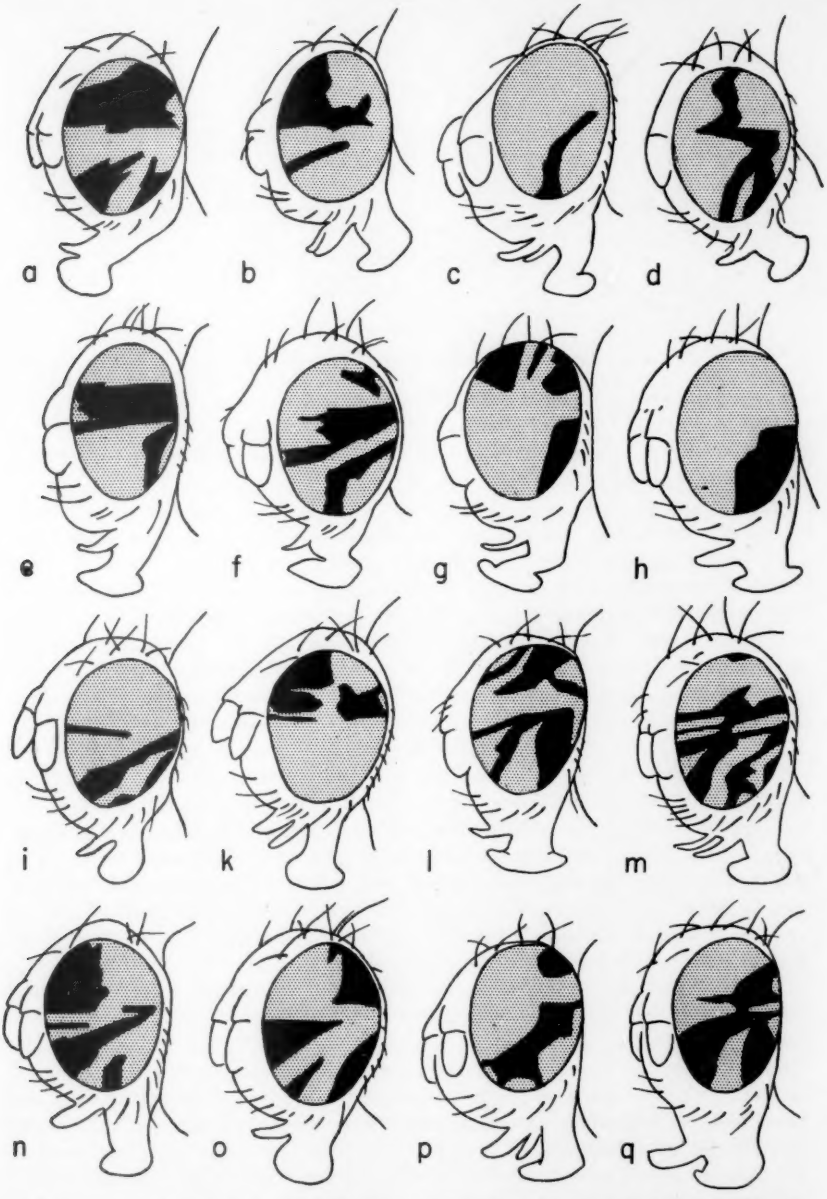

FIGURE 3.—Left eyes of z^m males. Black = c.l. spots; dotted = lemon background with salt-and-pepper mottling.

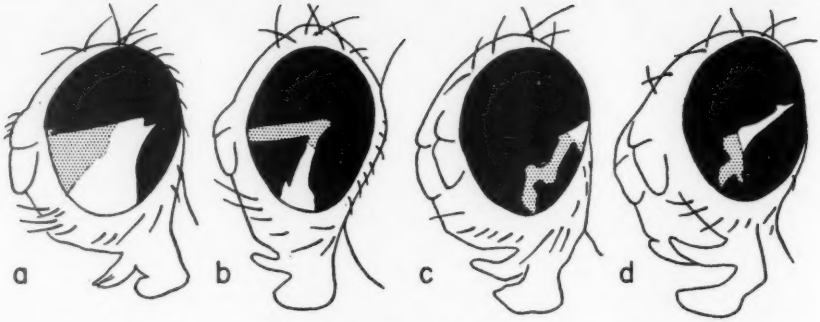

FIGURE 4.—Twin spots induced by X-irradiation of younger (a) and older (b-d) first instar larvae of the constitution *sc* z^m w^+/sc^+ z^+ *w*. Black = normal red; dotted = lemon partner (*sc* z^m w^+/sc z^m w^+), white = white partner (*sc*$^+$ z^+ *w*/*sc*$^+$ z^+ *w*) of mosaic spot.

(*sc* z^m w^+/sc z^m w^+) and one white (*sc*+ *z*+ *w*/*sc*+ *z*+ *w*) partner in an otherwise normally pigmented eye.

The shape and arrangement of large spots that were induced in the embryo and during the first larval instar showed a remarkable regularity, especially in the lower half of the eye. The spots in the upper half of the eye are not basically different but are not as regular in shape and position as the spots in the lower half. The reason for this difference is apparently that beginning with the end of the first larval instar, the growth centrum of the eye anlage is in the presumptive upper half of the eye, and growth in this center seems to be less regular than in parts of the anlage more distant from it. For this reason the analysis of the zeste alleles will be limited to the lower half of the eye.

It has been shown (BECKER 1957b) that the spots induced by treatment at the end of the first larval instar and earlier subdivided the lower half of the eye into roughly eight sectors. This was found when all these spots were drawn on the outlines of a single eye. A schematic representation of these results is given in Figure 5. Each one of the spots induced at the end of the first larval instar covers the area of one of the eight sectors, each one induced earlier covers the area of two or more of the sectors. The presumptive area for the lower half of the eye consists, therefore, at the end of the first larval instar, of about eight cells, and the presumptive total eye area consists of about 20 cells.

In the two alleles z^m and z^l the pattern of the variegation spots corresponds completely to the cell lineage pattern of X-ray-induced mosaic spots. It can be concluded, therefore, that those variegation spots are, like the mosaic spots, derivatives from single cells of the eye anlage. This means that in early development in z^m and z^l, some cells are determined to form lemon parts of the eye, and other neighboring cells are determined to form red parts of the eye. Whether or not this potential difference is realized depends on the temperature under which the flies are raised, as was discussed earlier. These large variegation spots will be referred to from now on as cell lineage or c.l. spots.

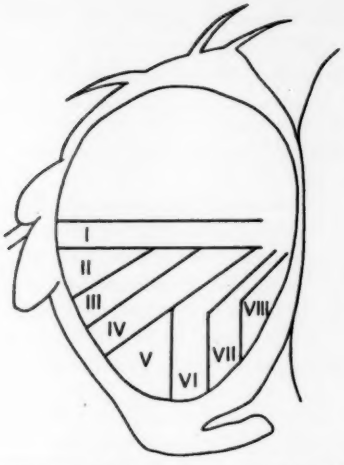

FIGURE 5.—Schematic representation of the shape and position of mosaic spots in the lower half of the eye, induced by irradiation at the end of the first larval instar. Earlier induced spots cover the area of two or more of the eight sectors.

Flies of the constitution z^m; *st/st* or z^l; *st/st* have almost white eyes with scarlet c.l. spots. The mutant scarlet (*st*) is known not to allow the formation of the brown pigment component. Apparently, therefore, the difference between the two cell types, i.e., those determined to form lemon and those determined to form red, is: some cells are able and some cells are not able—the appropriate temperature conditions provided—to form red pigment, the second of the two pigment components.

The stage at which cells are determined as to whether or not their descendants form red pigment should be given by the size of c.l. spots. The size of mosaic spots induced at different developmental stages and the size of c.l. spots have for this reason been compared.

The smallest c.l. spots of z^m and z^l eyes have the size equivalent to one of the sectors in Figure 5 (Figure 3b, c); all other spots have the size of two or more sectors. The smallest ones, then, are derived from cells determined at the end of the first larval instar. The larger ones could have been derived either from cells which were determined earlier or from a number of adjacent cells at the end of the first larval instar. To settle this question, 110 eyes of z^l males, each with a total red area of two sectors in the lower half of the eye, were rated as to whether they were adjacent or separate (compare Figure 3d–g). If in these cases the determination would have taken place at the "one sector stage", i.e., would have affected any two cells out of eight at random, one would have to expect—under the given condition of eight sectors in a row—that in 25 percent of the cases the two sectors would be adjacent (Figure 1g) and that in 75 percent of the cases the two sectors would be separate (Figure 1d–f). Among the 110 eyes investigated, 33

(30 percent) showed adjacent sectors and 77 (70 percent) showed separate sectors. This indicates that the determination preferably takes place at only one stage during development and, furthermore, that this stage is the end of the first larval instar.

The quantitative treatment of variegation in z^m and z^l in which the number of sectors covered by c.l. spots are counted provides an opportunity to make further distinctions between the alleles and their compounds. First, however, it has to be shown that the c.l. spot incidence is independent of culture conditions.

It is known from the work of CHEN (1948) that the temperature effective period for the differences of pigmentation controlled by the allele z (as given in Figure 1) is in the pupal stage. The same is true for z^m and z^l. This was shown by transferring larvae at different developmental stages from 25°C to 17°C and *vice versa*. Whenever pupae developed at 17°C the eyes of the flies looked like the 17°C controls; whenever pupae developed at 25°C the eyes looked like the 25°C controls.

Next an attempt was made to find out whether the number of sectors covered by c.l. spots, i.e., the number of cells that are determined for red pigment formation, can be influenced by temperature. The temperature effective period would be expected before or during the time of determination, i.e., the end of the first larval instar, which is approximately 40–42 hours after egg deposition.

The experiment was done with z^m males. After preliminary tests had shown that no differences could be expected from comparing 17 and 25°C as experimental temperatures, z^m males were uniformly raised at 25°C and transferred at different stages either for six days into 13°C or for three hours into 36°C. The first four rows of Table 1 show the result of two different cold treatments and one heat treatment. Out of all sectors investigated, i.e., eight sectors per eye, the percentage covered by a c.l. spot was determined and is given in the rightmost column of the table. This is identical to the percentage of anlage cells determined to red pigment formation. The number after the transfer to 13°C of 20-hour old stages lies slightly above the control value; the number after transfer of 30-hour old stages lies slightly below it but is not significantly different from it. The flies whose larvae were transferred 30 hours after egg deposition for three hours to 36°C show also a value slightly but not significantly below the control. z^m and probably z^l have, therefore, no temperature effective period at the end of the first larval instar, but they both do have the same pupal temperature effective period as was found for z.

Notwithstanding this apparent independence of the c.l. spot incidence from the temperature during the larval stages, all flies whose spots were counted later were uniformly raised at 25°C until the end of their larval life. Then they were transferred into the temperature in which c.l. spots develop most clearly, and that is given in the third column of Table 1. The detailed data on the distribution of eyes with various proportions covered by c.l. spots are given in Table 2.

z^m females show c.l. spots only when they are raised at low temperature. Table 1 shows that only 2–3 percent of their anlage cells become determined for red pigment formation (rows 5 and 6).

TABLE 1

The percentage of sectors in the lower half of the eye covered by cell lineage spots, for different genotypes and under different culture conditions

No. of row	Genotype	Culture conditions	No. of eyes investigated*	Total no. of c.l. spot sectors	Percent sectors (or cells)†
1	*sc* z^m/Y	25°C	608	1,149	23.6
2	*sc* z^m/Y	20h after egg deposition for 6 days 13°C	185	368	24.9
3	*sc* z^m/Y	30h after egg deposition for 6 days 13°C	142	249	21.9
4	*sc* z^m/Y	38h after egg deposition for 3 hours 36°C	284	498	21.9
5	*sc* z^m/*sc* z^m	17°C	2,510	400	2.0
6	*sc* z^m/*sc* z^m	14°C	490	114	2.9
7	(*y f*: = ×) *sc* z^l/Y	25°C	1,813	1,558	10.8
8	(*y f*: = ×) *sc* z^l/Y	17°C	280	214	9.6
9	*sc* z^l/Y	25°C	534	541	12.7
10	*sc* z^m/Y	both from *sc* z^m/*sc* z^l mothers; 25°C	912	1,663	22.9
11	*sc* z^l/Y		931	1,427	19.1
12	*sc* z^l/*sc* z^l	14°C	616	149	3.0
13	*y* z^a/ *z*	14 and 17°C	614	...	0.0
14	*y* z^a/*sc* z^m	14°C	748	124	2.1
15	*y* z^a/*sc* z^l	14°C	1,184	106	1.1
16	*z*/*sc* z^m	14°C	1,004	166	2.1
17	*z*/*sc* z^l	14°C	766	91	1.5
18	*sc* z^m/*sc* z^l	14°C	1,043	407	4.9
19	*sc* z^l/0	25°C	267	266	12.5

* Since each eye has eight sectors (see Figure 5), the total amount of sectors investigated is eight times the number of eyes investigated.

† Column 6 gives the percentage of sectors covered by c.l. spots. This percentage is at the same time the percentage of cells determined for red pigment formation.

The first z^l male found was crossed to double X females (*y f*:=) and kept with them for several generations before a homozygous z^l stock was established. Rows 7 and 8 of Table 1 show that in the genetic environment of this stock (*y f*:= × *sc* z^l) the average number of cells determined for red pigment formation (10.8 and 9.6) lies well under the value of z^m males (23.6). Row 9, on the other hand, shows that the value went up in males of the homozygous z^l stock (12.7). In order to be able to compare the c.l. spot incidence in z^m and z^l males in an identical environment and with identical genetic background, the male offspring of

TABLE 2

The distribution of eyes with various proportions covered by cell lineage spots

No. of row*	Number of sectors affected (percent)								
	0	1	2	3	4	5	6	7	8
1	29.5	20.8	18.1	12.7	7.6	6.1	3.5	1.5	0.5
2	22.2	26.5	23.2	10.3	7.0	3.8	2.7	2.7	1.6
3	28.2	29.6	14.8	12.0	4.9	4.9	4.2	0.7	0.7
4	28.8	25.7	22.5	7.4	4.6	4.6	3.2	3.2	..
5	88.3	8.0	2.9	0.6	0.0	0.0	..	..	..
6	83.0	12.0	4.5	0.4	..	0.2	..	..	..
7	52.5	25.3	12.1	6.2	2.0	1.3	0.3	0.1	0.0
8	59.2	20.4	10.7	6.4	1.4	1.1	0.7	..	..
9	48.5	26.4	12.4	5.6	3.4	2.8	1.1	..	..
10	33.0	23.0	14.6	11.0	7.0	4.3	3.4	2.3	1.3
11	36.5	24.2	16.1	10.3	5.4	3.2	1.8	1.6	0.8
12	81.3	14.3	3.7	0.8	..	..	..	..	..
13	100.0	..	..	..	..	..	..	..	..
14	90.5	5.1	2.4	1.5	0.4	0.1	..	..	..
15	94.1	3.6	1.5	0.4	0.3	..	..	..	..
16	89.6	6.0	2.8	1.1	0.4	..	..	..	..
17	93.2	4.0	2.1	0.4	0.1	0.1	..	0.1	..
18	73.2	18.4	5.5	1.8	0.7	0.2	..	..	..
19	46.1	27.4	15.0	6.4	3.7	0.7	0.7	..	..

* Row numbers correspond to those given in Table 1.

z^m/z^l compound females were raised at 25°C and investigated. Rows 10 and 11, Table 1, give the result. The difference is small (22.9 *vs.* 19.1), but still highly significant, using a contingency chi-square test for the sum of the data: $x^2 = 29.0$; $P < 0.001$. The eyes of z^m males are darker than the ones of their z^l brothers, because of their salt-and-pepper mottling (see Figure 1). That this difference in background color of the eyes is not a source of error can be seen from rows 7 and 8, Table 1, where the lighter z^l males (row 7) show even a slightly higher spot incidence (10.8) than the darker ones (9.6; row 8).

The investigated z^l females (row 12, Table 1) have a different genetic background from the z^m females and, therefore, cannot be compared exactly with them. In any case, both are very similar, i.e., 2–3 percent, so that in z^l, as in z^m, the spot incidence in females is very much lower than in males.

On the basis of the difference between z^m and z^l males (rows 10 and 11, Table 1) it was justified to expect a smaller average number of spots in z^l than in z^m females. That this might still be the case, identical genetic background provided, is suggested by the data of the compounds. In compounds with both z^a and z, z^m shows a somewhat higher spot incidence than z^l: z^a/z^m, 2.1 percent; z^a/z^l, 1.1 percent (rows 14 and 15, Table 1) and z/z^m, 2.1 percent; z/z^l, 1.5 percent (rows 16 and 17, Table 1). z^a/z flies show, as stated earlier, no c.l. spots at all (row 13, Table 1), while z^m/z^l females show a surprising increase in spots compared to the homozygotes of either allele: z^m/z^m, 2.9 percent; z^l/z^l, 3.0 percent; z^m/z^l, 4.9

percent. Again, however, they should be coisogenic before further comparison is made.

As M. GANS (1953) has shown, the Y chromosome has no influence on the salt-and-pepper mottling of *z* females. X0 males with the allele z^l were investigated in order to find out whether the Y chromosome influences the number of c.l. spots. The males were derived from a cross of attached-X females without Y chromosome to *sc* z^l males ($\underline{Y} \times sc\ z^l$). The result, 12.5, is much like the value of z^l males from the homozygous stock (12.7; row 9, Table 1). The absence of the Y chromosome, therefore, does not seem to influence the incidence of c.l. spots.

The distribution of c.l. spots on the eye

Those presumptive eye cells in the anlagen that are the predecessors of the lower half of the eye grow more regularly than those which form the upper half of the eye, as was stated earlier. It was for this reason that the quantitative scoring of c.l. spots was limited to the lower half of the eye. Judging from the general appearance of the eyes, there is no reason to assume that the percentage of cells that become determined for red pigment formation is different in the presumptive upper half of the eye. The difficulty of scoring in the upper half, however, is a hindrance in getting definite evidence for this statement.

The distribution of c.l. spots over the lower half of the eye is shown in Table 3, upper row. Three hundred and sixteen sectors, each covered by one or part of one c.l. spot, are apparently equally distributed over all eight sector positions. This shows that the determination affects the cells of the anlage randomly, at least in the presumptive lower half of the eye.

This action of the alleles z^m and z^l on cells at the end of the first larval instar is interestingly comparable to the action of the Lobe alleles. The data from a newly arisen Lobe allele (BECKER 1957b) are summed up in the second row of Table 3. When the flies were raised at 18°C, 35 percent of the eyes were normal, whereas in the other 65 percent the eyes had gaps very similar in size, shape and position to X-ray-induced mosaic spots or c.l. spots of z^m and z^l (Figure 6). The analysis showed that this Lobe allele acted at the same stage as z^m and z^l, i.e., at the end of the first larval instar. The analysis of both, X-ray-induced mosaic spots and the Lobe deformities, indicated that at this stage in larval life the ommatidia field of the anlage is created. One of the properties that could be assigned to this field was the position of its center and its minimal diameter for the develop-

TABLE 3

The distribution of cell lineage spots in z^m *and eye defects in Lobe over the different sectors of the lower half of the eye (in percent)*

Allele	Sector								Total sectors covered by spot (z^m) or affected by defect (Lobe)
	I	II	III	IV	V	VI	VII	VIII	
z^m	13.3	12.3	11.7	13.3	11.1	11.7	15.8	11.7	316
Lobe	19.7	17.5	15.3	12.6	11.2	8.4	7.9	7.2	2,100

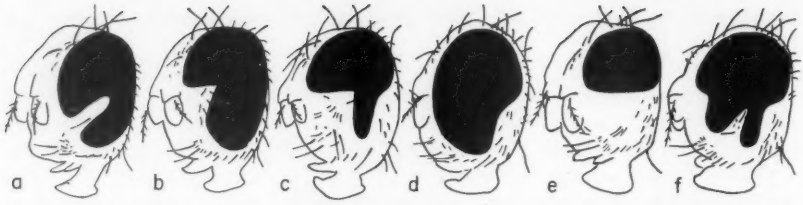

FIGURE 6.—Left eyes of the mutant Lobe, raised at 18°C (redrawn from BECKER 1957b).

ment of a normal eye. And the action of the investigated Lobe allele could be interpreted as a diminution of the field's diameter. The gradient that characterizes the distribution of 2100 registered defect sectors (Table 3, lower row) can be understood with the assumption that the field's center is laid down in that region of the anlage which forms the posterior central region of the eye. It should be pointed out that under these temperature conditions only the lower half of the eye is affected by the eye reduction.

One principal difference between the action of the zeste alleles z^m and z^l and the action of the Lobe allele is, then, that the determination of red pigment formation in the zeste alleles affects single anlage cells randomly, whereas the determination as to whether or not the descendants of a cell form ommatidia in Lobe eyes follows a gradient. Determination of cells in Lobe is apparently a part of the properties of a newly established, presumably centric ommatidia field. The action of both groups of alleles has, however, several common features. Both act at the same stage in larval life, and both determine at this stage the future fate of all the descendants of single cells. According to the size of the sectors and according to cell counts made of anlagen during the second larval instar (BECKER 1957b), about five cell divisions per cell take place between the time of determination at the end of the first larval instar and the time when the eye anlage consists of ommatidia stem cells in the third larval instar.

The gaps in the eyes of Lobe flies raised at 18°C do not distort the shape of the head. It is most likely, therefore, that all ommatidia stem cells keep growing at their normal rate. While ommatidia stem cells normally start with ommatidia formation by differential divisions, descendants of those anlage cells that failed to become determined for ommatidia formation do not start with differential division, but apparently divide into epithelial cells instead. These latter cells form later a normal kind of head cuticle that fills the gaps of the Lobe eyes.

The primary pigment cells of the ommatidia are the site where the red pigment is formed. They do not appear until four cell divisions after the beginning of ommatidia differentiation, i.e., about nine cell divisions after the determination for red pigment formation in z^m and z^l.

Thus, the conclusions that can be drawn so far are: In the zeste alleles z^m and z^l there are a certain percentage of eye anlage cells randomly determined as to whether or not their descendants, nine cell divisions later, will form red pigment. In Lobe the anlage cells are determined non randomly as to whether their de-

scendants start, after having undergone five cell divisions, with the ommatidia formation by differential divisions or divide into epithelial cells.

The action of the Lobe allele has to be understood, as was pointed out earlier, as diminution of the ommatidia field, i.e., as interference with a normal developmental process. The randomness of the z^m and z^l action, however, poses the question, whether the determination for red pigment formation at the end of the first larval instar is part of the normal development of the eye and whether, consequently, z^m and z^l interfere with such a process. It is possible to get at least a clue toward an answer. One can raise z/z^m or z/z^l heterozygotes (that develop normal eye color) until the second or third larval instar, and at this stage change single cells of the anlage into homozygous z^m/z^m or z^l/z^l cells. These changed cells, then, were heterozygous at the end of the first larval instar. If they had been determined at that time for normal red pigment formation, one would not expect to find any mosaic spots developing from these homozygous cells. If z^m/z^m or z^l/z^l cells induced as late as the third larval instar do form ommatidia with zeste phenotype, then there is apparently no determinative process concerning the eye pigmentation at the end of the first larval instar of normal flies.

To decide between these two alternatives, larvae heterozygous for z^m ($sc^+z^+/sc\ z^m$) were X-rayed. Their age was 65–75 hours after egg deposition, i.e., they were at the beginning of the third larval instar. The flies were checked for z^m/z^m mosaic spots. These spots were found in abundance. This means, then, that in normal cells no determination concerning the eye pigment takes place at the end of the first larval instar. In z^m, however, once the determination as to the type of pigmentation has been made, the cells keep the newly acquired property and hand it down to the daughter cells until the pigment becomes formed.

The zeste alleles interfere with the formation of the eye pigments. For the alleles z^m and z^l, in particular, the creation of the ommatidia field at the end of the first larval instar seems to be a critical point of interference. Once the action of z^m and z^l has been exerted, the cells hand down the new, abnormal property to their daughter cells. If a cell becomes homozygous for these alleles at any later stage of development, this genetic constitution still allows an interference with the normal process of pigment formation.

DISCUSSION

Up to this point the discussion has been driven as far as the observations and the experimental results warranted. In this section a comparison between our results and those of other workers will be made in an attempt to define the nature of the determination further.

The determination has always been referred to as a determination to form red pigment. All one can see, however, is that parts of the eye contain red pigment while others lack it. To be precise, one can only speak of the cells being determined to contain red pigment, as long as nothing exact is known about the properties by which cells of a c.l. spot differ from the other cells. The possible properties might differ in the capability to form pigment, to take up pigment into the

cells or to incorporate pigment into the pigment granules within the cells. Since pigment formation is one of the possibilities and almost nothing is known about the others, the zeste action will be discussed in connection with some known facts about red pigment formation.

The work of HADORN and others (see HADORN 1958) revealed the first case of a gene dependent enzyme in Drosophila, xanthine dehydrogenase. This enzyme was found to be present in normal larvae as early as the first larval instar and is for the most part produced in the Malpighian tubules and the fat body. It is missing in flies homozygous for the eye color mutant rosy. The enzyme is involved in the formation of isoxanthopterine, a precursor of the red pigment. In spite of the early presence of the enzyme, isoxanthopterine is produced only in the pupal stage. Thus the enzyme is formed very early in development, and either the enzyme itself or the pigment precursor is able to be transported to the site of their interaction. The production of the pigment precursor takes place right at the time when the eye becomes pigmented. If the absence of red pigment in zeste eyes would be due to the absence of this enzyme, one certainly could not expect any c.l. type of variegation.

There are, however, other mutants that interfere in a different way with the production of red pigment. In most of these cases the interfering mutant acts autonomously, i.e., in mosaics each cell develops a phenotype according to its own genotype. The reason for this could be that other enzymes or pigment precursors are incapable of diffusing or of being transferred from one cell to another. One would, however, be more inclined to assume that those parts of the reaction chain leading to red pigment, with which the autonomous mutants interfere, are taking place at a stage in development when transportation from normal cells to mutant cells is not possible any more.

Under such an assumption the determination in z^m and z^l eye anlagen at the end of the first larval instar does not seem to be of a kind that endows some cells with a fixed substance and not others. One would have to think of the determination rather in terms of endowment of the cells with different functional potentialities. These potentialities would have to be attached to certain cellular entities. It is at present not possible to say what cellular constituent might be the site of the determination.

In other lines of investigations one finds cases that offer themselves as models for an interpretation of the present findings. Thus, in their nuclear transplantation work in *Rana pipiens* KING and BRIGGS (1956) and BRIGGS and KING (1957) showed that stabilized changes of the capacity for differentiation take place in nuclei of developing endoderm. In another example, the localized swellings (puffs) in giant chromosomes of Dipterans are believed to be forms of differential activation of chromosome loci (BEERMANN 1959). In some of these cases rather stable changes take place in connection with the formation of a puff (PAVAN and BREUER 1955). In *Drosophila melanogaster* under experimental conditions, a puff has even been induced at a chromosome locus where puffing normally has not been observed (BECKER 1959). This puff appears in some cells of a gland and not in others.

Because of the close resemblance to the variegation in the zeste alleles mention must be made also of variegation due to chromosome rearrangements in which heterochromatin is involved. In eyes of *Drosophila virilis*, BAKER (1953) found a variegation pattern apparently identical to that of the c.l. spots of the zeste alleles z^m and z^l; in his case the heterochromatic peach locus had been transposed into euchromatin. Many other effects of heterochromatin on variegation are reviewed by SCHULTZ (1939), LEWIS (1950) and HANNAH (1951). Of special interest in connection with the zeste variegation are more recent findings by SCHULTZ (1956). He found in a variegating white translocation that an irreversible differentiation of pigment in cells of Malpighian tubules occurs in the embryonic stage. In another case where the Stubble locus is transferred into heterochromatin, it could be shown that the action of heterochromatin on the variegation of the locus is involved with the nucleic acid metabolism. The extent of the heterochromatin-dependent variegation is even paralleled by the degree of heterochromatization of salivary chromosome bands carrying the respective genes (SCHULTZ 1941).

All the cases mentioned above have in common with the variegation phenomenon of z^m and z^l the phenomenon of stable changes in somatic cells; and in all those cases the site of the change was the nucleus. Similarly, the site of differential determination for red pigment formation in z^m and z^l could also be the nucleus.

SUMMARY

Of the four zeste alleles z, z^a, z^m and z^l, the two latter ones show a variegation pattern characteristic for cell lineage (c.l.) in both males and females, and also in compounds with the two former alleles. In flies raised in favorable temperatures, which are different for each allele and each sex, the c.l. spots are normal red or slightly darker than normal; they contain both the red and the brown pigment component. The background of the eye is lemon and salt-and-pepper mottled or only lemon; in this latter case the red pigment component is missing.

The size and distribution of the c.l. spots were compared with X-ray-induced mosaic spots, whose size depends on the larval age at the time of irradiation.

It can be concluded that the time of determination as to whether the descendants of a cell develop red pigment or not takes place at the end of the first larval instar. Of the approximately 20 cells of which the presumptive eye area consists at this time of development, any number can be determined to red pigment formation in any individual head anlage, on the average 20–25 percent in z^m males, 10–20 percent in z^l males, 2–3 percent in z^m/z^m and z^l/z^l females and none, 2.1 percent, 1.1 percent, 2.1 percent, 1.5 percent and 4.9 percent in z^a/z, z^a/z^m, z^a/z^l, z/z^m, z/z^l and z^m/z^l compounds respectively. The site of the red pigment formation, the primary pigment cells, does not appear until about nine cell divisions after the determination.

The action of the zeste alleles is discussed and compared with the action of the eye-deforming mutant Lobe.

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LITERATURE CITED

BAKER, W. K., 1953 V-type position effects of a gene in *Drosophila virilis* normally located in heterochromatin. Genetics **38**: 328–344.

BECKER, H. J., 1957a Zur Entwicklungsphysiologie des Drosophila-Auges. Verh. Dtsch. Zool. Ges., Hamburg, 1956: 256–264.

1957b Über Röntgenmosaikflecken und Defektmutationen am Auge von Drosophila und die Entwicklungsphysiologie des Auges. Z. Ind. Abst. Vererb. **88**: 333–373.

1959 Die Puffs der Speicheldrüsenchromosomen von *Drosophila melanogaster*. I. Beobachtungen zum Verhalten des Puffmusters im Normalstamm und bei zwei Mutanten, *giant* und *lethal-giant-larvae*. Chromosoma **10**: 654–678.

BEERMANN, W., 1959 Chromosomal differentiation in insects. *Developmental Cytology*. pp. 83–103. Edited by D. RUDNICK. The Ronald Press Co. New York.

BRIGGS, R., and T. J. KING, 1957 Changes in the nuclei of differentiating endoderm cells as revealed by nuclear transplantation. J. Morph. **100**: 269–311.

CHEN, S. Y., 1948 Action de la température sur trois mutants à panachure de *Drosophila melanogaster*: w^{253-18}; w^{m5} et *z*. Bull. Biol. France et Belg. **82**: 114–129.

GANS, MADELEINE, 1953 Étude génétique et physiologique du mutant *z* de *Drosophila melanogaster*. Bull. Biol. France et Belg. (Suppl.) **38**: 1–90.

HADORN, E., 1958 Role of genes in developmental processes. pp. 779–791. *The Chemical Basis of Development*. Edited by W. D. MCELROY and B. GLASS. The Johns Hopkins Press. Baltimore, Md.

HANNAH, A., 1951 Localization and function of heterochromatin in *Drosophila melanogaster*. Advances in Genet. **4**: 87–125.

KING, T. J., and R. BRIGGS, 1956 Serial transplantation of embryonic nuclei. Cold Spring Harbor Symposia Quant. Biol. **21**: 271–290.

LEWIS, E. B., 1950 The phenomenon of position effect. Advances in Genet. **3**: 73–115.

PAVAN, C., and M. E. BREUER, 1955 Differences in nucleic acid content of the loci in polytene chromosomes of *Rhynchosciara angelae* according to tissues and larval stages. Symp. "Cell Secretion", Belo Horizonte, Brazil: 90–99.

SCHULTZ, J., 1939 The function of heterochromatin. Proc. 7th Intern. Congr. Genet. 257–262.

1941 The evidence of the nucleoprotein nature of the gene. Cold Spring Harbor Symposia Quant. Biol. **9**: 55–65.

1956 The relation of the heterochromatic chromosome regions to the nucleic acids of the cell. Cold Spring Harbor Symposia Quant. Biol. **21**: 307–328.

STUDIES OF ADENYLOSUCCINASE IN MUTANTS AND REVERTANTS OF NEUROSPORA CRASSA[1]

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MANY investigations of mutation in both forward and reverse directions have been made during the past three decades (see Brookhaven Symposia in Biology, 1955). Most of the earlier experiments were performed with Drosophila (PATTERSON and MULLER 1930; TIMOFÉEFF-RESSOVSKY 1933; KAUFMANN 1942; LEFEVRE 1950) or maize (STADLER 1944; STADLER and ROMAN 1948) and utilized gross morphological phenotypes as criteria for determining whether or not a particular gene had undergone complete reverse mutation. More recently, investigations have been made employing nutritional mutants of microorganisms (RYAN and LEDERBERG 1946; GILES and LEDERBERG 1948; KØLMARK and WESTERGAARD 1949; GILES 1951, 1956; GILES, DE SERRES and PARTRIDGE 1955; GLOVER 1956; DE SERRES 1958). Nutritional mutants permit the detection of certain intermediate mutational states at specific loci by means of accumulated intermediates, differential growth rates, etc. Where the genetic control over specific enzymes is known, measurements of enzyme activity in revertants have provided a means of distinguishing among revertants which are phenotypically similar in their growth rates on minimal medium. In a study of 15 ultraviolet-induced reverse mutants from an *ad-4* (adenylosuccinaseless) mutant in Neurospora (GILES, PARTRIDGE and NELSON 1957), all revertants were found to have a level of adenylosuccinase activity less than that of the wild type. Quantitative measurements of glutamic dehydrogenase activity in back mutants derived from one of the *am* (glutamic acid dehydrogenase deficient) alleles in Neurospora (PATEMAN 1957) revealed that seven of 28 back mutants possessed less enzyme activity than the original wild type. The assay procedure, however, did not permit a distinction among revertants having subnormal levels of enzyme activity nor among any of those apparently equivalent to wild type. Recent study of tryptophan-independent revertants in *E. coli*, derived from mutants lacking tryptophan synthetase activity (STADLER and YANOFSKY 1959; YANOFSKY and CRAWFORD 1959), shows that both suppressor mutation and reverse mutation occur. Qualitative as well as quantitative distinctions at the enzyme level were made between partial revertants and wild type.

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Previous reports have indicated the complexity of the *ad-4* locus, which controls the synthesis of adenylosuccinase in Neurospora (GILES, PARTRIDGE and NELSON 1957; WOODWARD 1958; GILES 1958; WOODWARD, PARTRIDGE and GILES 1958; WOODWARD 1959). The present study represents an attempt to analyze several mutant phenotypes at the enzyme level. From this study, it is apparent that enzyme differences may result from either forward or reverse mutational changes at this locus.

Terminology

The terms reversion and revertant are used in this paper to indicate any adenine-independent isolate arising from an *ad-4* mutant (GILES 1951). When reverse mutation is specified, genetic tests have indicated a change at or very near the *ad-4* locus. By this definition, all *ad-4* revertants analyzed genetically to date have resulted from reverse mutation rather than from suppressor mutation.

Primary mutants are mutants derived directly from wild type, and primary revertants are revertants obtained directly from primary mutants. Similarly, secondary mutants are derived from primary revertants and secondary revertants, from secondary mutants.

Genetic markers utilized in this study were: *leu* (*leu-1*), a leucine requiring mutant; *leu*$^+$, a leucine-independent allele of *leu-1*; *pan-2*, a pantothenic acid requiring mutant.

MATERIALS AND METHODS

All Neurospora mutants used in this investigation were obtained from a 74A St. Lawrence wild type stock or from strains that were derived by inbreeding of 74A. Mutant F23 is of spontaneous origin; all other primary mutants were isolated from macroconidia treated with 36,000r of X-rays. The assay procedure for making adenylosuccinase activity determinations (spectrophotometric measurement of the disappearance of adenosine monophosphate succinate [AMP-S]) has been described (GILES, PARTRIDGE and NELSON 1957). This procedure was modified in the present experiments by the substitution of Tris (tris [hydroxymethyl] aminomethane) HCl for phosphate buffer except where otherwise noted. All assays were performed at 35.5°C.

Reverse mutation experiments were performed with double mutants (*pan-2 ad-4*) in all cases. *ad-4* revertants were isolated by plating heavy suspensions of irradiated or nonirradiated conidia from such double mutants onto a minimal medium containing pantothenic acid. This procedure eliminates to a considerable extent the possibility of recovering undetected contaminant wild types and screens selectively for reversions to adenine independence. Only *ad-4*$^+$ colonies carrying the *pan-2* marker were saved, and these were routinely backcrossed to the parental *ad-4* mutant to obtain homocaryotic *ad-4*$^+$ *pan-2* strains. Single ascospore isolates were then used for subsequent enzyme assays. Assays of a given revertant with or without the *pan* marker agree as well as replicate assays of a culture from a single spore isolate. Revertants obtained from secondary mutant Y203-M1 were not backcrossed. In this instance, revertants were assayed directly

for adenylosuccinase activity because backcrossing difficulties were encountered; presumably most such revertants were heterocaryotic.

Primary and secondary *ad-4* mutants (WOODWARD, PARTRIDGE and GILES 1958) were obtained by the filtration and selective plating method (WOODWARD, DEZEEUW and SRB 1954). It was not necessary initially to test the growth response of all the colonies isolated, since *ad-4* mutants are distinguishable by the accumulation of a slight amount of purple pigment in the conidia when adenine in the medium is limiting.

RESULTS

Quantitative studies of adenylosuccinase in revertants: In this investigation, quantitative levels of adenylosuccinase activity were measured in extracts of a large number of revertants obtained from eight different *ad-4* alleles. Five of the eight alleles are shown in the complementation map of the *ad-4* locus (Figure 1),

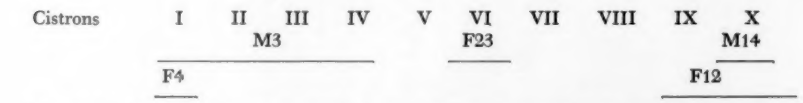

FIGURE 1.—Complementation map of the *ad-4* locus showing the relationship that exists among the mutants used in this investigation. Mutants F2, F20 and M1 are noncomplementing types, hence are not shown here.

which illustrates the pattern of complementation characteristic of each allele (WOODWARD, PARTRIDGE and GILES 1958). The other three alleles are noncomplementing mutants.

The degree of restoration of adenylosuccinase activity in the revertants varies, and appears to depend primarily on the allele from which each was obtained. The frequency distribution of activities in revertants is not a continuous one, however, since among the 112 revertants examined, only a limited number of fairly discrete quantitative categories are represented (Figure 2). The diagram in Figure 3 (together with Table 1 and Figure 2) indicates the origin and relationships of primary and secondary revertants used in this investigation. For example, mutant F12 has yielded 38 revertants with approximately 25 percent of wild type enzyme activity, nine with three percent of wild type activity, and one revertant with ten percent of wild type activity. In most cases, the same quantitative categories are observed among revertants of spontaneous origin as among ultraviolet- or X-ray-induced revertants (Table 1). To insure independence of origin for F12 revertants of spontaneous origin, each spontaneous revertant was isolated from a separate mutant culture.

The only class of revertants characterized by growth rates less than that of wild type on an adenine-free medium is the group exhibiting only three percent of wild type enzyme activity. These revertants show only slightly subnormal growth rates, but the reality of these differences can be established by the increases in growth rates observed upon supplementation with adenine (Table 2).

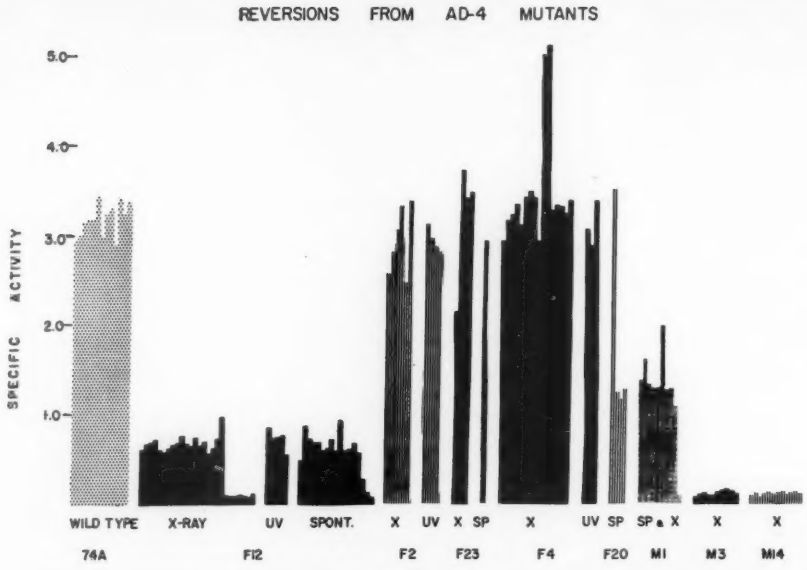

FIGURE 2.—Adenylosuccinase activities in revertants of independent origin. The eight *ad-4* alleles used yielded revertants spontaneously and/or following X-ray or ultraviolet irradiation. Specific activity was measured as $\Delta A_{280}/2.8$ mg dry wt/20 min/0.77 ml (see Table 3 for full details). Common parental mutant (in bottom line) for a revertant group is indicated by common marking pattern of bars representing that group.

Two primary reverse mutants from F12 gave rise to several secondary mutants. The primary reverse mutant having three percent of wild type enzyme activity was used to produce two secondary mutants (M3 and M14), from which 20 secondary revertants were derived. All of these possessed approximately three percent of wild type activity. On the other hand, the primary F12 reverse mutant having 25 percent of wild type enzyme activity gave rise (through secondary mutant M1) to secondary revertants possessing a higher level of enzyme activity than their parental primary reverse mutant, as well as to others in the three percent category (Figures 2 and 3).

Genetic studies of revertants: Previous reverse mutation studies at the *ad-4* locus showed that only four of 21 mutants tested failed to yield adenine-independent colonies following mutagenic treatment (NELSON 1957). None of the revertants investigated appeared to involve suppressor mutations as judged by an analysis of crosses in which loosely linked or nonlinked suppressors would have been detected (GILES 1958). The mutants that do revert to adenine independence appear to have characteristic reverse mutation rates. Such differences in reverse mutation rates are probably allele specific and presumably indicate that mutability isoalleles are present at the *ad-4* locus, as has been shown to be true at other

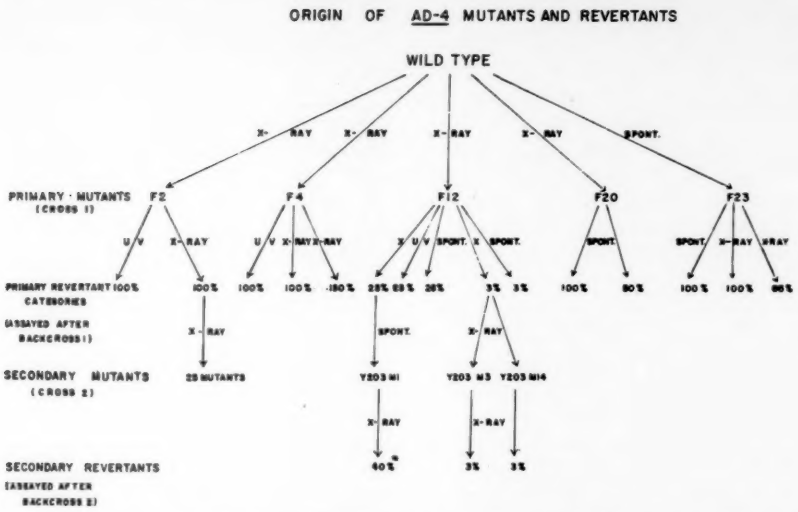

FIGURE 3.—Derivation of primary and secondary revertants. All *ad-4* mutants were crossed (crosses 1 and 2) to a *pan-2* mutant (unlinked marker). Double mutants (*ad-4 pan-2*) were used for the production of revertants in all cases. All primary and most secondary revertants were backcrossed (backcrosses 1 and 2) to their parental *ad-4* mutants to obtain homocaryotic stocks. Percentage figures indicate the approximate specific adenylosuccinase activities of the quantitative categories of revertants obtained, expressed as percent of wild type activity.

* Revertants from Y203 M1 were not backcrossed before assay.

loci in Neurospora (GILES 1951; GILES, DE SERRES, and PARTRIDGE 1955). However, definitive tests employing allelic crosses having linked markers have not yet been performed.

Genetic information has been obtained from crosses of revertants selected from different quantitative categories with respect to adenylosuccinase activity. In general, the results of such crosses, employing linked markers, indicate that the quantitative differences in adenylosuccinase activities characteristic of individual revertants are inherited as allelic differences at the *ad-4* locus (GILES 1958). Additional data supporting this view are presented in Tables 3 and 4. The relatively high activity values (averaging about 50 percent) for the two F12 revertants indicated in Table 4 are similar to results obtained in previous studies employing mycelial extracts prepared in phosphate buffer (GILES, PARTRIDGE, and NELSON 1957). This effect of the extraction buffer on adenylosuccinase activity will be discussed further in the next section of this paper.

The present results, together with those presented previously from both ordered and random ascospore isolations from crosses of *ad-4* revertants with wild type (GILES 1958), provide evidence that substantially all *ad-4* reversions arise by reverse mutation rather than by suppressor mutation. In addition, the tetrad data from crosses of revertants having subnormal levels of adenylosuccinase ac-

TABLE 1

Classification of ad-4 *revertants according to their adenylosuccinase activity relative to that of wild type (74A)*

Primary mutants	Pattern of complementation	Mutagen used in obtaining revertants	Quantitative categories represented in percent	No. rev./ category
F2	none	X-ray	100	6
		U. V.	100	4
F4	Cistron I	X-ray	100	7
		X-ray	150	2
		U. V.	100	3
F12	Cistrons IX and X	X-ray	3	7
		X-ray	25	20
		U. V.	25	5
		none	3	2
		none	10	1
		none	25	14
F20	none	none	35	3
		none	100	1
F23	Cistron VI	X-ray	65	1
		X-ray	100	3
		none	100	1
Secondary mutants	(derived from F12 revertants)			
M14	Cistron X	X-ray	3	12
M3	Cistrons I, II, III and IV	X-ray	3	10
M1*	none	X-ray	3	1
		X-ray	35	9

See Figure 1 for complementation map of mutants used to obtain revertants. See Figure 2 for individual revertant activities.

* Revertants from this mutant were not backcrossed to obtain homocaryotic single spore isolates due to crossing difficulties.

tivity indicate that modifying factors at other loci are of relatively minor importance in affecting the levels of enzyme activity in various revertants.

Only two cases have been found in which the quantitative level of enzyme activity appears to involve modifiers at other loci. One of these is a primary revertant of F4, Y201-R24, which possesses 150 percent of wild type adenylosuccinase activity. Table 5 shows the segregation of high enzyme activity in what appears to be a random fashion with respect to the *leu-1* marker which has been mapped about two units to the right of the *ad-4* locus (NELSON 1957). The other revertant that seems to be influenced by modifiers was derived from mutant F12. In this instance, only three cultures obtained from random spore isolations have been assayed. These assays gave the widely differing values of 12.7, 15.8, and 21.0 percent of wild type enzyme activity. In all other revertants that were studied, the results from separate spore cultures agree as closely as separately grown cultures of the same isolate (see Table 3).

Complementation studies with reverse mutants: Attempts have been made to detect complementation between reverse mutants (derived from either the same or different alleles) having subnormal levels of enzyme activity. In no case was a synergism or inhibition detected; the heterocaryons merely showed an additive effect of the two separate enzyme activities. These heterocaryons were forced by using nutritional mutant markers which would not permit growth on the minimal medium without heterocaryosis. It has been possible, however, to detect complementation between revertants having three percent of wild type activity and certain complete mutants (WOODWARD 1959). Such complementation resulted in a level of enzyme activity in the heterocaryon approximating 25 percent of wild type.

Qualitative differences between enzymes from reverse mutants and wild type: Preliminary tests indicate that there are qualitative differences associated with the enzymes extracted from reverse mutants having subnormal levels of adenylosuccinase activity. Furthermore, the tests that have been made indicate that all of the revertants from a single quantitative category contain an AMP-S splitting enzyme with similar properties. For example, the revertants with three percent of wild type activity are also characterized by a lag of five minutes or longer before the maximal rate of reaction is attained. A similar lag is observed among revertants with 25 percent of wild type activity, but in addition, the specific activity of revertants in this category is increased to approximately 50 percent of wild type by extracting in pH 7.0 phosphate buffer instead of the usual pH 8.0 Tris HCl

TABLE 2

Linear growth rates of F12 revertants at 25°C on supplemented and unsupplemented agar medium (in horizontal test tubes)

Revertant number	Approximate adenylosuccinase activity (percent of W.T.)	Growth in mm/hr over 30 hr period		
		Minimal	150 μg/ml adenine	Difference
74A wild type	100	3.94	4.00	+0.06
3	25	3.59	3.62	+0.03
4	25	3.68	3.62	−0.06
9	25	3.75	3.88	+0.13
15	25	3.78	3.84	+0.06
18	25	3.47	3.45	−0.02
19	25	3.70	3.68	−0.02
25	25	4.00	3.91	−0.09
26	25	3.94	3.94	0.00
10	3	3.23	3.68	+0.45
11	3	2.49	2.64	+0.15
13	3	3.13	3.68	+0.55
17	3	3.78	4.06	+0.28
20	3	3.15	3.47	+0.32
24	3	3.43	4.00	+0.57
28	3	3.02	3.83	+0.81
30	3	3.37	3.91	+0.54

TABLE 3

Segregation of subnormal levels of adenylosuccinase activity

Cross	Phenotype	Ascus and spore no.	Relative activity*	Percent of wild type activity
Y201–R9 × *leu-1*	leu	3.1	3.39	105.5
	leu	3.3	3.46	108.0
	leu$^+$	3.5	1.43	44.5
	leu$^+$	3.7	1.34	42.0
Y191–R14 × *leu-1*	leu$^+$	1.1	0.78	24.5
	leu$^+$	1.2	0.85	26.5
	leu	1.3	4.24	132.5
	leu	1.4	4.12	130.0
	leu$^+$	1.5	0.71	22.0
	leu$^+$	1.6	0.80	25.0
	leu	1.7	3.88	121.0
Y191–R28 × *leu-1*	leu	2.1	2.72	85.0
	leu	2.2	3.20	100.0
	leu	2.3	3.26	102.0
	leu	2.4	3.02	94.5
	leu$^+$	2.5	0.19	5.9
	leu$^+$	2.6	0.14	4.4
	leu$^+$	2.7	0.16	5.0
	leu$^+$	2.8	0.15	4.7
Y191–R14 × Y191–R13	leu$^+$	4.1	0.16	5.0
	leu$^+$	4.2	0.16	5.0
	leu$^+$	4.3	0.74	23.0
	leu$^+$	4.4	0.76	23.5
	leu$^+$	4.5	0.77	24.0
	leu$^+$	4.6	0.76	23.5
	leu$^+$	4.7	0.15	4.7
	leu$^+$	4.8	0.14	4.4

* Measured as ΔA_{280}/20 min/2.8 mg dry wt/0.77 ml total volume with excess substrate at 35.5°C in pH 8.4, 0.05 *M* Tris HCl; extracted in pH 8.0, 0.05 *M* Tris HCl after three days growth at 25°C (Standard conditions).

extraction buffer (Table 6). (Tris buffer of pH 7.0, employed as a control in this experiment, eliminated pH as the factor responsible for the large differences observed.) Furthermore the lag in the reaction observed when the enzyme is extracted in Tris buffer does not appear when the enzyme is extracted in phosphate. The lag is not eliminated by the addition to the Tris-buffer-extracted enzyme of pyrophosphate, phosphate, sodium or potassium, or combinations of these. No differences in specific activity have been observed when other classes of revertants or the wild type strain have been extracted in the two different buffers.

An F23 revertant with about 65 percent of wild type enzyme activity can be distinguished from wild type by comparing reaction rate (decrease in absorption) *vs.* time (Figure 4). The revertant enzyme requires about 3.5 minutes to attain its maximum reaction rate and the rate begins to decrease at a higher residual substrate concentration than is the case with the wild type enzyme.

One of the revertants of F12, in the 25 percent activity class, (Y 191-R4), was subjected to further qualitative tests. It had been noted in a preliminary survey that this was one of the few strains which produced an enzyme differing in substrate specificity from the wild type (GILES 1958). Therefore, other conditions were sought which would further characterize it qualitatively. Some of the same tests were also carried out on another similar revertant of F12 (Y219-R1) and on revertant progeny of R4. In general, it appears that the revertant enzymes are

TABLE 4

Adenylosuccinase activity of segregants from crosses of UV-induced back mutants F12–R164 and F12–R165 with a leucine-requiring strain (leu-1)

Expt. no.	Back mutant parent	Ascus and spore no.	Phenotype	Relative activity* (percent leucine parent)
1	R164	1.1	leu+	50
		1.4	leu+	50
		1.6	leu	88
		1.8	leu	102
	R164	5.1	leu	80
		5.4	leu+	50
		5.5	leu+	40
		5.7	leu	86
	R165	41.1	leu	78
		41.4	leu	82
		41.5	leu+	40
		41.7	leu+	44
	R165	43.2	leu	98
		43.4	leu+	38
		43.5	leu+	31
		43.8	leu	77
	Controls:	630–2a (*leu-1*)	leu	98
		630–1A (*leu-1*)	leu	102
		R164	leu+	52
		R165	leu+	47
2	R164	5.1	leu	121
		5.4	leu+	51
		5.5	leu+	58
		5.7	leu	103
	R165	42.2	leu	93
		42.4	leu	108
		42.5	leu+	58
		42.8	leu+	50
	Controls:	630–2a (*leu-1*)	leu	100
		630–1A (*leu-1*)	leu	89
		R164	leu+	61

* Enzyme activity measured with excess substrate at 35.5°C in pH 8.4, 0.05 *M* Tris HCl; 1:11 dilution of pH 7.0 0.05 *M* phosphate extract of 40 mg mycelium/ml; 69 hours growth at 25°C.

TABLE 5

Segregation of high adenylosuccinase activity

Cross	Phenotype	Ascus and spore	Relative activity*	Percent of wild type activity
Y201–R24 × *leu-1*	leu$^+$	3.1	2.86	91.0
	leu$^+$	3.3	4.25	133.0
	leu	3.5	3.02	94.5
	leu	3.7	7.15	223.0
	leu$^+$	5.2	3.76	118.0
	leu	5.4	3.32	104.0
	leu$^+$	5.6	4.36	136.5
	leu	5.8	3.48	109.0
	leu	6.1	...	.....
	leu$^+$	6.3	2.67	83.5
	leu$^+$	6.5	4.32	135.0
	leu	6.7	3.18	99.5

* Standard conditions (see Table 3).

more sensitive to inhibitory or deleterious treatments than is the wild type enzyme. Furthermore these differences are greater when the substrate used is SAICAR (N-[-5-amino-1-ribosyl-4-imidazolecarbonyl]-L-aspartic acid 5′-phosphate) than when it is AMP-S, as will be discussed later.

The revertant enzymes are much more thermolabile than is wild type enzyme; incubation at 35°C for seven minutes, while having no effect on wild type enzyme, destroys 90 to 100 percent of the activity of the revertants. However, this sensitivity disappears in the presence of glutathione (10^{-3}M). In fact, glutathione (with or without preincubation) increases the rate of AMP-S splitting by R4 by 33 percent, while having little effect on wild type activity. (The spectral changes in the course of the reaction were not altered qualitatively by the addition of glutathione).

Revertant activity is also inhibited by zinc at a concentration much lower than that affecting the wild type. R4 was chosen for further examination of the influence of metal ions and a procedure was adopted involving a 7.25 minute preincubation of the extract with the chloride salt of the metal in the presence of 10^{-3} molar glutathione, before addition of substrate. Constant maximal reaction rates were compared with controls identical except for the omission of metal salts. At the 10^{-5} molar level, divalent zinc and copper both inhibit the splitting of AMP-S by 65 percent, while divalent nickel and cobalt and trivalent chromium and iron produce little or no inhibition. Wild type is not inhibited by either copper or zinc at these levels. An identical test of R4 action on SAICAR showed a similar zinc inhibition (71 percent), again with no inhibition of wild type.

In a series of four asci from two crosses, the pairs of R4 progeny (carrying parental markers) which gave the highest and lowest activities on both AMP-S and SAICAR (cf. GILES 1958) were tested (as above) for zinc inhibition on

AMP-S. The most active pair was least inhibited (by 65 percent) and the least active pair was most inhibited (by 85 percent) by 10^{-5} molar zinc (Table 7). In all these progeny specific activities for both substrates were invariably much closer to those of the parent carrying the same genetic marker than to those of the other parent, indicating the allele specificity of the enzymic activity.

Comparisons of substrate specificities: When the existence, in the same biosynthetic sequence, of a second natural substrate for the single enzyme, adenylosuccinase, was discovered (BUCHANAN *et al.* 1957; GILES, PARTRIDGE, and NELSON 1957; GOTS and GOLUB 1957), tests were made on representative complementary *ad-4* mutants to determine whether their behavior might be based on differences

TABLE 6

Comparison of adenylosuccinase activities of representative revertants with Tris vs. *phosphate extraction buffers*

Strain	Relative activity*	Extraction buffer	pH	Percent of W.T. activity
74A (wild type)	3.16	Tris HCl	8.0	100.0
	3.10	Phosphate	7.0	100.0
F12–Y191–R19	0.98	Tris HCl	8.0	31.0
	1.46	Phosphate	7.0	47.2
	1.10	Tris HCl	7.0	34.3
F12–Y167–R168	0.73	Tris HCl	8.0	23.1
	1.74	Phosphate	7.0	56.2
F12–Y167–R166	0.85	Tris HCl	8.0	27.4
	1.74	Phosphate	7.0	56.2
F12–Y167–R184	0.74	Tris HCl	8.0	23.4
	1.63	Phosphate	7.0	52.6
F12–Y167–R186	0.76	Tris HCl	8.0	24.0
	1.42	Phosphate	7.0	45.8
F12–Y167–R156	0.54	Tris HCl	8.0	17.1
	1.46	Phosphate	7.0	47.2
F12–Y191–R28	0.09	Tris HCl	8.0	2.85
	0.065	Phosphate	7.0	2.10
F4–Y164–R128	3.48	Tris HCl	8.0	111.0
	3.34	Phosphate	7.0	107.5
F2–Y164–R15	3.04	Tris HCl	8.0	96.3
	3.30	Phosphate	7.0	106.5
F12–Y209–R1	0.79	Tris HCl	8.0	24.7
	1.03	Tris HCl	7.0	32.2
F4–Y201–R23	3.36	Tris HCl	8.0	105.0
	3.24	Tris HCl	7.0	101.0

* Standard conditions (see Table 3).

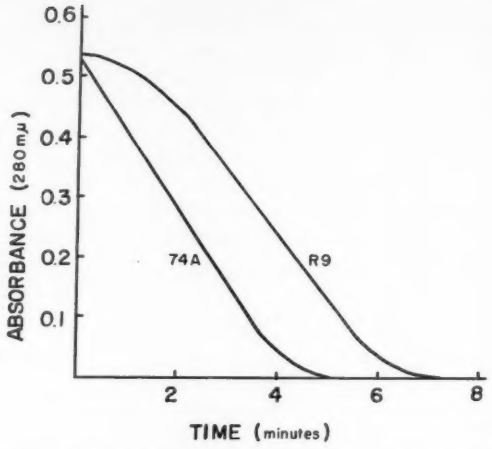

FIGURE 4.—Continuous tracing of the decrease in absorbance at 280 mμ. The wild type (74A) extract was diluted to give approximately the same maximal rate of decrease in absorbance as the extract from F23 revertant Y210–R9.

TABLE 7

Adenylosuccinase activity of segregants from crosses of reverse mutant Y191-R4 with a leucine requiring strain (leu-1) *and with its parental mutant (F12)*

Cross no.	Parents	Ascus and spore no.	Phenotype	Substrate: AMP-S Relative activity (percent W.T.)	Substrate: SAICAR Relative activity (percent W.T.)	Substrate: AMP-S (+ 10^{-3} *M* glutathione ± 10^{-5} *M* $ZnCl_2$) percent inhibition by $ZnCl_2$
1	R4 × *leu-1*	9.1	leu	100	119	..
		9.4	leu	99	160	..
		9.5	leu+	21	15	62
		9.7	leu+	22	15	67
		10.1	leu+	21	14	..
		10.4	leu+	20	13	..
		10.5	leu	98	119	..
		10.7	leu	110	132	..
2	R4 × F12	2.1	ad+	18	6	78
		2.4	ad+	20	6	88
		3.1	ad+	22	9	..
		3.4	ad+	18	7	..
	R4	..	ad+	23	7	62
	leu-1	..	leu	100	112	..

Standard conditions of growth, extraction and assay, except for addition in last column, as noted.

in the specificities of their activities for the two substrates AMP-S and SAICAR (GILES, PARTRIDGE, and NELSON 1957; GILES 1958). No such differences were found.

Additional observations have provided further evidence that adenylosuccinase is a single enzyme. Even in the case of a revertant possessing specific activities twice the normal level (derived from mutant F4), the relative substrate specificity was similar to that of wild type. Furthermore, in a chromatogram (on DEAE cellulose, with salt gradient elution) of a thermolabile adenylosuccinase (from the temperature-sensitive mutant 44206 = F15) the activity ratio for the two substrates remained constant throughout the series of active eluate fractions, as was the case in BUCHANAN's purification of the normal enzyme from yeast (MILLER, LUKENS, and BUCHANAN 1959). It was also observed that the enzyme produced by an interallelic heterocaryon (F4 + F23) showed a wild type ratio of activities for the two substrates and was no more sensitive to zinc than was the normal enzyme, even after chromatographic purification.

However, despite the fact that most strains are not distinguishable from wild type in substrate specificity, when a number of revertants were examined, several were found to possess definitely less activity for SAICAR than for AMP-S (expressed in percentage of wild type specific activity). One of these (R4) was selected for further study; although certain preliminary results of this study were reported previously (GILES 1958), these observations have now been extended further with the results reported here.

Under ordinary assay conditions the revertant enzyme splits SAICAR at a relatively much slower rate than AMP-S, but a constant velocity is maintained from the beginning of the reaction, without the lag or activation period observed on AMP-S. The addition of glutathione eliminates these differences. That is, the reaction velocity of SAICAR increases to a constant value, after about 20 minutes, which is 245 percent greater than its rate without glutathione. The AMP-S reaction rate is increased by only 33 percent by glutathione (or by 2, 3-dimercaptopropanol). Thus, activation of the revertant enzyme with glutathione can greatly reduce if not eliminate the differential in relative reaction velocities of the two substrates, in addition to removing their differences in substrate activation ability. It has also been observed (using derived R4 strains) that a twofold increase in initial AMP-S concentration over the standard level doubles the constant maximum reaction velocity attained after the usual lag period. This substrate activation effect is rapidly reversible, as evidenced by a renewal of the lag on addition of more substrate after the end of the period of constant velocity.

In a further attempt to determine whether mutational changes can produce *ad-4* alleles with clear differences in relative substrate specificities, a procedure has been devised to detect revertants having the ability to split AMP-S but not SAICAR. This procedure involves plating ultraviolet- or X-ray-treated conidia of various *ad-4* mutants on a medium—minimal supplemented with hypoxanthine—which does not support the growth of the original mutants. Colonies which appear are tested for growth on unsupplemented medium, in a search for mutants which can carry out the later but not the earlier biosynthetic function of adeny-

losuccinase. Among 115 isolates tested, from two different *ad-4* mutants, no such differential revertants were found. That is, none would grow on hypoxanthine without growing on minimal medium also.

DISCUSSION

The existence of complete reverse mutation has been viewed with some skepticism (GOLDSCHMIDT 1955). Complete reverse or return mutation is taken to mean that the mutant gene in question has been changed back to a structure in every way identical with its original configuration. To decide this matter definitely would require the elucidation of the precise molecular structure of a given gene. A much less precise but, at present, more practical substitute would appear to be an examination and comparison of gene products, such as a particular enzyme under the control of a given locus. The ultimate test, even at the gene product level would require a detailed structural chemical analysis; i.e., an amino acid sequential analysis, or the detection of differences in secondary or tertiary structure if other tests failed to distinguish between the enzymes produced by the reverse mutant and the wild type.

The approach described in the present paper involves a less definitive characterization of the gene product; nevertheless, distinctions can be made between the wild type enzyme and the enzymes produced by revertants having subnormal levels of enzyme activity. It appears significant that no qualitative difference has been detected between the wild type enzyme and the enzyme produced by reverse mutants having quantitative levels of enzyme activity equivalent to wild type (utilizing the same tests that permitted a distinction in the other cases). Studies on other enzymes (YURA 1959; SUSKIND, YANOFSKY and BONNER 1955) suggest that a subnormal level of enzyme activity may indicate only a qualitative rather than a quantitative difference in the enzyme in question.

The genetic data presented in this paper make it evident that most of the mutations studied—those occurring in both the forward and reverse directions—are associated with changes at the *ad-4* locus. On the basis that the mutational changes, as judged by alterations in the quantitative and/or qualitative measurements of the enzyme adenylosuccinase, are indeed reflections of changes in the molecular structure at the *ad-4* locus, the results of this investigation can be interpreted to indicate that a limited number of possible molecular configurations will result in repair of this locus, thus restoring adenylosuccinase activity. Some mutations lead to the formation of a protein which, although capable of carrying out the splitting reaction, differs in a quantitative and/or qualitative way from the wild type enzyme. The number of categories of revertants obtained varies with different mutant alleles. From F12, two distinct categories were recovered (3 percent and 25 percent) with a possible third category represented by the one revertant with ten percent of wild type enzyme activity. The fact that most of these quantitative categories are also separable on the basis of qualitative comparisons at the enzyme level supports the validity of the quantitative classification. Nonetheless, in spite of the similarities that are observed between enzymes

obtained from reverse mutants within a single quantitative category, the possibility exists that they may differ in more subtle ways that have not yet been detected. Such changes could reflect very small differences that are incapable of detection by the methods used, or they could be changes that are a matter of indifference to the enzyme as far as its activity is concerned. Actually, some evidence was found for qualitative differences in enzyme behavior between the "25 percent group" of revertants as a whole and at least one of its members, as will be discussed later. However, the fact that only a limited number of distinguishable types of reverse mutants has been obtained from a given allele suggests that only a limited number of changes can result in repair, either partial or complete, of the damage incurred at the time of the original forward mutational event.

Previous studies (NELSON 1957) have indicated that the damage incurred by forward (direct) mutation at the *ad-4* locus may be either reversible or irreversible, since certain alleles are apparently incapable of reverse mutation. In addition, the present studies have demonstrated that certain alleles, although capable of yielding revertants, apparently possess another type of irreversible damage, since they cannot undergo complete reverse mutation, as judged by the characteristics of the adenylosuccinase produced by revertants occurring in such mutants. In the case of the primary mutant, F12, only revertants with subnormal levels of enzyme activity have been obtained. Furthermore, a second type of irreversible change has apparently occurred in those primary revertants (from F12) with only three percent of wild type enzyme activity. This change is revealed when secondary mutants from three percent primary revertants are used to produce adenine-independent (secondary) revertants. Of 20 such secondary revertants assayed, all possessed approximately three percent of wild type enzyme activity. The 20 revertants assayed were selected from over 100 revertants on the basis of having the most rapid growth rates. On the other hand, a primary revertant (from F12) with 25 percent of wild type enzyme activity has been used to obtain secondary revertants possessing a higher level of activity than their parental primary revertant.

One F4 revertant having a high level of enzyme activity (150 percent of wild type) is interesting in that reverse mutation appears to have occurred at the *ad-4* locus in conjunction with an independent mutation at a loosely-linked or nonlinked locus. Both of these mutational changes seem to be involved in the attainment of the supernormal level of enzyme activity. When this modifier gene is crossed into wild type (employing crosses with markers closely linked to the *ad-4* locus), the modifier affects such a strain in a like manner; i.e., the level of enzyme activity is raised to over 150 percent of normal wild type. No qualitative differences have been detected between the enzymes from the revertant having a supernormal level of activity and from normal wild type. It may be noted in this connection that certain other strains possessing wild type *ad-4* alleles (ad^+), including some related to 74A and some unrelated strains, have yielded similarly high adenylosuccinase activities. Enzyme repressor genes of the type well-established in bacteria may be involved here.

Another instance of what may be modifier effects is seen (in Table 7) in the

quantitative and qualitative variations between revertant progeny in separate asci from crosses of a single revertant (R4). It therefore appears that factors in the genetic background as well as components of the assay medium (e.g. glutathione) may exert a marked influence on the degree of deviation of such revertants from normal in enzymatic assay behavior. These observations suggest the need for further evidence before concluding that indications of heterogeneity within a quantitative category of revertants (e.g., the relative substrate specificity within the 25 percent group) reflect real differences in the structure of the enzyme and its controlling locus. The possibility must be entertained that closely-linked modifiers may be responsible for altering the tertiary structure of the enzyme (during growth or during extraction) to different degrees in the various members of the quantitatively intermediate class of revertants. Whether these differences are expressed in the behavior of the enzymes in their native state *in vivo* remains problematical. At any rate, procedures devised for detecting absolute differences in substrate specificities of the enzyme during growth of induced revertants have not yet provided any evidence of such cases.

It seems probable that the degrees of difference in substrate specificity thus far observed *in vitro* are secondary reflections of differences in the condition of the enzyme molecules, as extracted. Such a situation may lead to varying degrees of reduction in the general efficiency and stability of enzyme molecules which are identical in their primary structure, these deviations being magnified in the presence of SAICAR as substrate as well as by generally unfavorable conditions during extraction and assay. The greater effect of AMP-S in activating the revertant (R4) enzyme, as compared with SAICAR (especially in the absence of added glutathione), may be connected with its more rigid structure and/or its possession of an extra carbon atom. This revertant form of adenylosuccinase may represent an extreme example of the requirement of enzymes in general for activation through alteration of surface configuration by contact with substrate, as KOSHLAND (1959) postulates.

In spite of these reservations concerning the interpretation of observed qualitative differences in the adenylosuccinase activity within one quantitative category of revertants, when the whole series of revertants is considered, the evidence clearly supports the attribution of the various qualitative and quantitative abnormalities in adenylosuccinase activity, characteristic of the different groups of revertants, to a variety of reverse mutational changes at the *ad-4* locus. Even within the restricted group just discussed, the differences have not been eliminated by crossing procedures, leaving the existence of associated allelic and enzymatic differences within major quantitative categories of revertants as the currently favored interpretation.

The present investigations are also of interest in connection with the question of whether reverse mutations can be induced by X-rays (cf. LEFEVRE 1950). Although certain *ad-4* alleles are apparently incapable of reverse mutation, the majority can revert either spontaneously, or following ultraviolet or X-ray treatment. Furthermore, there is no evidence for marked differences in the categories of reverse mutants induced in a given allele by various treatments. For certain

alleles (e.g., F2, F23, and F4), present evidence indicates that X-rays are capable of producing reverse mutants equivalent to the original wild type on the basis of both genetic analysis and enzyme assays. Thus, these results agree with evidence obtained at other loci in Neurospora (GILES 1951, 1956), in particular with those studies performed by DE SERRES (1958) with *ad-3* mutants. Recent experiments also indicate that reverse mutations can be induced by X-rays at certain loci in Drosophila (GREEN 1959).

Although these results indicate that X-rays can induce reverse mutation of certain *ad-4* alleles, there remains the related question of whether mutants initially produced by X-rays can back mutate. In the experiments reported here, general evidence supports the view that essentially all the *ad-4* mutants (including those capable of X-ray-induced reverse mutation) derived from macroconidia exposed to X-rays were indeed induced by the X-irradiation. This conclusion is supported by the fact that the yield of mutants was very much higher in irradiated conidia than in untreated conidia from parallel control experiments. Specific reconstruction experiments designed to eliminate the possibility that selection of pre-existing *ad-4* mutants might occur in the X-ray experiments were not performed. However, such experiments have been performed by DE SERRES (1958) with *ad-3* mutants and his results strongly support the view that adenine mutants derived from macroconidia exposed to X-rays and recovered by the filtration-concentration procedure have indeed been induced by the X-ray treatment.

SUMMARY

Revertants have been obtained from eight different *ad-4* (adenylosuccinaseless) mutants. Most of the mutants were derived from X-irradiated conidia, while revertants were obtained from untreated, X-irradiated, or ultraviolet-treated condia. Adenylosuccinase activity has been restored to at least some degree in all revertants. All present evidence indicates that the revertants studied are true back mutants. Suppressor mutations affecting the *ad-4* locus have not been found.

Reverse mutants derived from a given allele can be categorized according to the level of restored adenylosuccinase activity. There appear to be a limited number of quantitative levels of enzyme activity to which a given allele is capable of being restored by reverse mutation. Evidence is presented for certain qualitative differences in the enzymes obtained from revertants of different quantitative categories, while such differences have not been detected between enzymes from revertants within a single category. Within one such category, however, qualitative differences have been found by a different criterion, namely that of relative specificity for the two natural substrates, under certain conditions.

Whether or not a given *ad-4* allele is capable of reverting to a condition equivalent to wild type in terms of its level of restored enzyme activity appears to depend on the type of damage produced by the previous mutational event or events giving rise to the allele. Four out of five primary *ad-4* alleles yielded some reverse mutants quantitatively and qualitatively equivalent to wild type by the genetic and enzymological tests employed. Since the evidence indicates that at least some

of these *ad-4* mutants were of X-ray origin and that X-rays can produce reverse mutants indistinguishable from wild type, these results support the view that X-rays can induce complete reverse mutation of mutants originally produced by X-rays.

Additional mutational studies were performed with the one allele (F12) which failed to yield any primary revertants equivalent to wild type. These studies involved the production of secondary *ad-4* mutants from such primary *ad-4* revertants by forward mutation, and the subsequent induction of secondary reversions in such secondary mutants. The results indicate that secondary revertants derived from primary revertants which are restricted in that they produce an enzyme having a subnormal amount of activity are in some cases similarly restricted, while in other cases such secondary revertants surpass the primary revertants in the level of restored enzyme activity.

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LITERATURE CITED

BUCHANAN, J. M., J. G. FLAKS, S. C. HARTMAN, B. LEVENBERG, L. N. LUKENS, and L. WARREN, 1957 The enzymatic synthesis of inosinic acid *de novo*. pp. 233–255. *Ciba Foundation Symposium on the Chemistry and Biology of Purines*. Edited by G. E. W. WOLSTENHOLME and C. M. O'CONNOR. J. and A. Churchill Ltd. London.

DE SERRES, F. J., JR., 1958 Studies with purple adenine mutants in *Neurospora crassa*. III. Reversion of X-ray-induced mutants. Genetics **43**: 187–206.

GILES, N. H., 1951 Studies on the mechanism of reversion in biochemical mutants of *Neurospora crassa*. Cold Spring Harbor Symposia Quant. Biol. **16**: 283–313.

1956 Forward and back mutation at specific loci in Neurospora. Brookhaven Symposia Biol. **8**: 103–125.

1958 Mutations at specific loci in Neurospora. Proc. 10th Intern. Congr. Genet. **1**: 261–279.

GILES, N. H., F. J. DE SERRES, and C. W. H. PARTRIDGE, 1955 Comparative studies of X-ray induced forward and reverse mutation. Ann. N.Y. Acad. Sci. **59**: 536–552.

GILES, N. H., and E. Z. LEDERBERG, 1948 Induced reversions of biochemical mutants in *Neurospora crassa*. Am. J. Botany **35**: 150–157.

GILES, N. H., C. W. H. PARTRIDGE, and N. J. NELSON, 1957 The genetic control of adenylosuccinase. Proc. Natl. Acad. Sci. U.S. **43**: 305–317.

GLOVER, S. W., 1956 A comparative study of induced reversions in *Escherichia coli*. *Genetic Studies with Bacteria*. Carnegie Inst. Wash. Publ. **612**: 121–136.

GOLDSCHMIDT, R. E., 1955 *Theoretical Genetics*. pp. 105–107. Univ. of Calif. Press. Berkeley and Los Angeles.

GOTS, J. S., and E. G. GOLUB, 1957 Sequential blockade in adenine biosynthesis by genetic loss of an apparent bifunctional deacylase. Proc. Natl. Acad. Sci. U.S. **43**: 826–834.

GREEN, M. M., 1959 Radiation induced reverse mutations in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S. **45**: 16–18.

KAUFMANN, B. P., 1942 Reversion from roughest to wild type in *Drosophila melanogaster*. Genetics **27**: 537–549.

KØLMARK, G., and M. WESTERGAARD, 1949 Induced back mutations in a specific gene of *Neurospora crassa*. Hereditas **35**: 490–506.

KOSHLAND, D. E., JR., 1959 Mechanisms of transfer enzymes. pp. 305–346. *The Enzymes*. Second edition, Vol. 1. Edited by P. D. BOYER, H. LARDY, and K. MYRBÄCK. Academic Press. New York.

LEFEVRE, G., 1950 X-ray induced genetic effects in germinal and somatic tissue of *Drosophila melanogaster*. Am. Naturalist **84**: 341–365.

MILLER, R. W., L. N. LUKENS, and J. M. BUCHANAN, 1959 Biosynthesis of the purines. XXV. The enzymatic cleavage of N-(5-amino-1-ribosyl-4-imidazolylcarbonyl)–L-aspartic acid 5'-phosphate. J. Biol. Chem. **234**: 1806–1811.

NELSON, N. J., 1957 Genetical and biochemical investigations of adenine specific mutants in *Neurospora crassa*. Unpublished Ph.D. thesis. Yale University.

PATEMAN, J. A., 1957 Back mutation studies at the *am* locus in *Neurospora crassa*. J. Genet. **55**: 444–455.

PATTERSON, J. T., and H. J. MULLER, 1930 Are "progressive" mutations produced by X-rays? Genetics **15**: 495–577.

RYAN, F. J., and J. LEDERBERG, 1946 Reverse mutation and adaptation in leucineless Neurospora. Proc. Natl. Acad. Sci. U.S. **32**: 163–173.

STADLER, J., and C. YANOFSKY, 1959 Studies on a series of tryptophan-independent strains derived from a tryptophan-requiring mutant of *Escherichia coli*. Genetics **44**: 105–123.

STADLER, L. J., 1944 The effect of X-ray upon dominant mutation in maize. Proc. Natl. Acad. Sci. U.S. **30**: 123–128.

STADLER, L. J., and H. ROMAN, 1948 The effect of X-rays upon mutation of the gene A in maize. Genetics **33**: 273–303.

SUSKIND, S. R., C. YANOFSKY, and D. M. BONNER, 1955 Allelic strains of Neurospora lacking tryptophan synthetase: A preliminary immunochemical characterization. Proc. Natl. Acad. Sci. U.S. **41**: 577–582.

TIMOFÉEFF-RESSOVSKY, N. W., 1932 Mutations of the gene in different directions. Proc. 6th Intern. Congr. Genet. **1**: 308–330.

WOODWARD, D. O., 1958 Heterocaryon complementation between adenylosuccinaseless mutants of *Neurospora crassa*. Proc. 10th Intern. Congr. Genet. **2**: 321.
1959 Enzyme complementation *in vitro* between adenylosuccinaseless mutants of *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **45**: 846–850.

WOODWARD, D. O., C. W. H. PARTRIDGE, and N. H. GILES, 1958 Complementation at the *ad-4* locus in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **44**: 1237–1244.

WOODWARD, V. W., J. R. DE ZEEUW, and A. M. SRB, 1954 The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia, followed by filtration and selective plating. Proc. Natl. Acad. Sci. U.S. **40**: 192–200.

YANOFSKY, C., and I. P. CRAWFORD. 1959 The effects of deletions, point mutations, reversions and suppressor mutations on the two components of the tryptophan synthetase of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **45**: 1016–1026.

YURA, T., 1959 Genetic alteration of pyrroline-5-carboxylate reductase in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **45**: 197–204.

STUDIES WITH PURPLE ADENINE MUTANTS IN NEUROSPORA CRASSA. IV. LACK OF COMPLEMENTATION BETWEEN DIFFERENT ad-3A MUTANTS IN HETEROKARYONS AND PSEUDOWILD TYPES

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IN Neurospora a useful method of screening for alleles in a series of mutants with identical biochemical requirements is the test for complementation in heterokaryons. Mutants that complement in such tests and enable the heterokaryon to grow on minimal medium are considered nonallelic and are assumed to result from mutation at different genetic loci; whereas mutants that do not complement are considered allelic and are assumed to result from mutation at the same locus. The simplicity of this test has been somewhat complicated by the demonstration of allelic complementation at a number of loci (MITCHELL and MITCHELL 1956; FINCHAM and PATEMAN 1957; GILES, PARTRIDGE and NELSON 1957; CASE and GILES 1958a,b; CATCHESIDE and OVERTON 1958; LACY and BONNER 1958; LACY 1959; WOODWARD, PARTRIDGE, and GILES 1958). The identity of such complementing allelic mutants is not completely obscured by this interaction; however, such complementation is usually quite different from that obtained with nonallelic mutants. The latter grow at wild-type rate, whereas the former generally grow more slowly. The identity of complementing allelic mutants is also apparent in intercrosses since prototrophs are recovered at very low frequencies (10^{-3} or lower) or not at all, and the origin of such prototrophs shows no strict correlation with an exchange of markers in adjacent regions. In the analysis of *ad-3* mutants (DE SERRES 1956) quite different results were obtained. Whereas at other loci only a small proportion of the mutants show allelic complementation (see CATCHESIDE and OVERTON 1958), all the *ad-3* mutants studied were capable of apparent allelic complementation and all the heterokaryons grew at wild-type rate. Moreover, the origin of prototrophs in crosses between members of the two complementing groups of mutants showed a definite correlation with marker exchange. Such evidence was interpreted as indicating that these two groups of *ad-3* mutants were not identical, and that in this region purple adenine mutants resulted from mutation of two separate but closely linked loci (*ad-3A* and *ad-3B*) rather than one. In more comprehensive analyses of the mutants at each locus, no further evidence for complementation was found in any pairwise combination within each series.

Recently the accumulation of many new *ad-3* mutants has made it possible to investigate the phenomenon of complementation in this region in more detail.

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The data presented here are from a series of experiments to test for complementation among 13 different *ad-3A* mutants. Tests were made in forced and unforced heterokaryons and in pseudowild types (PWT's).

Strains

The *ad-3A* mutants A1-A10 and *ad-3B* mutant B17 were derived from the St. Lawrence wild-type strain 74A and are isogenic. Mutants A4-A10 were supplied by DR. MARY E. CASE, Yale University. The strains of *ad-3A* mutants 38701, 38709, and 68306 (BEADLE and TATUM 1945) were selected F_2 progeny from backcrosses to 74A. All 13 *ad-3A* mutants were classified as alleles on the basis of preliminary heterokaryon tests with *ad-3A* and *ad-3B* tester strains and their behavior in crosses with various *ad-3B* mutants (to be presented in detail in a subsequent paper). The *hist-2 ad-3A*(2) *nic-2* strain has been described previously (DE SERRES 1956).

The symbols used to refer to series of nonallelic mutant strains with the same biochemical requirement (i.e., *ad-1*, *ad-2*, *ad-3*, *ad-4*, etc.—adenine-requiring) are those proposed by BARRATT, NEWMEYER, PERKINS, and GARNJOBST (1954). The modified symbols *ad-3A* and *ad-3B* have been used to indicate further complexity within an apparently allelic series (DE SERRES 1956). Individual mutant designations which denote the position of a given mutant in the *ad-3* region used interchangeably in this paper are as follows: *ad-3A*(1) or A1; *ad-3A*(38701) or 38701; *ad-3B*(2) or B2, etc. The symbols used to designate mutant strains with other biochemical requirements are as follows: *hist*, histidine-requiring; *nic*, niacin-requiring; *pan*, pantothenate-requiring; *inos*, inositol-requiring; *pyr*, pyrimidine-requiring; and *am*, α-amino nitrogen-requiring.

Heterokaryon analyses

Heterokaryon tests with genetically unmarked strains: Initially, tests for complementation among the 13 *ad-3A* alleles were made with genetically unmarked strains as described previously (DE SERRES 1956). Heavy conidial suspensions ($1–5 \times 10^8$ conidia/ml) from each mutant were combined in all possible combinations, two at a time, on petri plates containing minimal agar. Combinations with *ad-3B* mutant B17 were included to ensure that all 13 *ad-3A* strains were of the appropriate genotype with reference to genes controlling heterokaryon formation and maintenance (GARNJOBST 1955). Within 24 hours at 27° C all *ad-3A* + *ad-3B* combinations formed heterokaryons with wild-type linear growth rate, which showed that all strains were fully compatible. All *ad-3A* combinations, however, were negative even after 5–7 days' incubation. Beyond this time in control experiments in which inoculum size and other aspects of the experimental procedure were varied, wild-type growth was observed with certain *ad-3A* combinations. At times both the heterokaryon and one of the homokaryotic controls grew, thus implicating reversion rather than allelic complementation. But, when no growth of either homokaryon was apparent, it was necessary to test for the possibility of reversion and the presence of adenine-independent nuclei. In such cases the tests were always positive, clearly indicating that such delayed growth

was caused by reversion of one of the homokaryons rather than allelic complementation. In a number of replicate experiments genetically unmarked strains of these 13 mutants were used, and there was no evidence of allelic complementation in any pairwise combination.

Since the mutants used in these tests were genetically unmarked and the presence of two different *ad-3A* mutant nuclei in the same cytoplasm cannot be readily proved, the possibility was considered that such negative evidence might be inconclusive. In theory, negative tests could also result from (1) some type of protoplasmic incompatibility (GARNJOBST and WILSON 1956); (2) disproportionate nuclear ratios attributable to inadequate nuclear mixing (PITTENGER and ATWOOD 1956); or (3) insufficient interaction between presumably defective gene products to produce functionally active enzyme (GILES, PARTRIDGE and NELSON 1957; FINCHAM and PATEMAN 1957; WOODWARD, PARTRIDGE and GILES 1958). To test these alternative interpretations of the negative results, a more comprehensive series of experiments was planned using forced heterokaryons between genetically marked strains. Such marked strains permit identification of different *ad-3* mutants in the same cytoplasm as well as precise control of nuclear ratios.

Selection and tests with genetically marked strains: Each of the 14 *ad-3* strains and wild-type strain 74A was crossed to a double mutant strain (319-OR5-8a) *inos* (inositol) *pan-2* (pantothenate) to obtain mating type A *inos* and A *pan-2* (referred to hereafter as *inos* and *pan*) derivatives of each mutant containing the same factors controlling heterokaryon formation and maintenance. From the cross of 74A, *inos* and *pan* strains were set up as standards and *ad-3 inos* and *ad-3 pan* derivatives of each mutant were tested with these two standard strains in forced heterokaryons on minimal medium in growth tubes to ensure that all derivatives were fully compatible. Linear growth rates of such forced heterokaryons were compared with the heterokaryon *inos* + *pan* and isogenic wild type strains of both mating types (74A and 74-OR8-1a). The data in Table 1 indicate that all such heterokaryons grow at wild type rate and that the *inos* and *pan* derivatives of each mutant have the same heterokaryon factors. The tests also show the complete dominance of the *ad-3* wild type alleles over each of the 14 *ad-3* mutant alleles used in the present experiments in heterokaryons.

Tests for complementation between genetically marked strains on minimal medium: To test for complementation in *ad-3A* + *ad-3A'* combinations, conidial suspensions of equivalent concentration of each of the 15 *pan* derivatives and the 15 *inos* derivatives were mixed two at a time on minimal medium supplemented with 0.1 mg of adenine/liter to establish forced heterokaryons in each of the 225 combinations. Conidia from each heterokaryon were tested in liquid minimal medium and in adenine-supplemented medium. Growth on the adenine-supplemented medium confirmed the presence of heterokaryotic macroconidia in each heterokaryon. Growth on minimal medium indicated complementation between the *ad-3* combinations. Since the average nuclear number per conidium is reduced by growing strains of Neurospora on minimal medium (HUEBSCHMAN 1952), the nuclear ratio of genetically different *ad-3A* mutant nuclei in heterokaryotic conidia should be approximately equal. None of the forced heterokaryons between

TABLE 1

Linear growth rate of forced heterokaryons of genetically marked ad-3 *mutants on minimal medium as compared with various wild-type strains*

Forced heterokaryons	Mean growth rate (cm/hr, 28°–32°C)	Forced heterokaryons	Mean growth rate (cm/hr, 28°–32°C)
A1 I + P	0.47	38701 I + P	0.48
A1 P + I	0.47	38701 P + I	0.47
A2 I + P	0.48	38709 I + P	0.48
A2 P + I	0.47	38709 P + I	0.48
A3 I + P	0.48	68306 I + P	0.48
A3 P + I	0.48	68306 P + I	0.49
A4 I + P	0.48		
A4 P + I	0.48	Controls	Mean growth rate (cm/hr, 28°–32°C)
A5 I + P	0.47	B17 I + P	0.49
A5 P + I	0.48	B17 P + I	0.46
A6 I + P	0.48	I + P	0.47
A6 P + I	0.48	74A	0.48
A7 I + P	0.50	74a	0.48
A7 P + I	0.49		
A8 I + P	0.48		
A8 P + I	0.48		
A9 I + P	0.48		
A9 P + I	0.49		
A10 I + P	0.47		
A10 P + I	0.48		

different *ad-3A* mutants grew on minimal medium, but conidia from all heterokaryons grew on the adenine-supplemented medium. The *ad-3A inos* + *ad-3B pan*, *ad-3A pan* + *ad-3B inos*, *ad-3 inos* + *pan*, and *ad-3 pan* + *inos* combinations also grew on minimal medium. In tests where there is no question of the presence of genetically different *ad-3A* nuclei in the same cytoplasm in almost equal nuclear ratios, no evidence for *ad-3A* complementation was found in any pairwise combination tested.

Tests for complementation on adenine-supplemented medium: The experiments of WOODWARD, PARTRIDGE, and GILES (1958) showed a very precise correlation of the time required for complementation to occur and of the level of complementation with the distance separating a given pair of *ad-4* alleles on a complementation map. Heterokaryons between mutants in adjacent cistrons require more time for the initiation of the complementation reaction and grow more slowly than heterokaryons between mutants separated by a number of cistrons. WOODWARD suggested that complementation results from a random exchange of defective gene products at either the RNA or polypeptide level in the cytoplasm

to yield enzymically active protein. He observed that the time required for growth of slow-growing heterokaryons to commence could be shortened within certain limits by increasing conidial concentration in the inoculum. Since similar variation in conidial concentration (up to about 5×10^8 conidia/ml) was without effect on forced or unforced heterokaryons of the 13 *ad-3A* mutants in the present study, tests of another type were developed to see if such negative results were attributable not to the complete lack of formation of functional enzyme but to formation at a low rate. If the rate of formation barely exceeds the degradation rate, it might permit, at a maximum, only very slow growth. With the use of suboptimal supplementation, the rate of linear growth of *ad-3* heterokaryons can be controlled precisely. The formation of functional enzyme when different *ad-3A* mutant nuclei are present in a common cytoplasm should result in a more rapid linear growth rate than with either mutant type alone. If the growth of heterokaryons containing complementing *ad-3A* mutants is similar to the growth of partial *ad-3B* revertants on minimal medium (DE SERRES 1958), we might expect a long lag period of very slow growth followed by a progressive increase in linear growth rate until wild-type growth rate is finally attained.

The rates of linear growth obtained with *ad-3A* mutants with suboptimal levels of supplementation are given in Figure 1. The range of the growth rates of forced heterokaryons of each strain (e.g., A1 *inos* + A1 *pan*) are given for each level tested. The variation in linear growth rates obtained with different mutants in such tests is the same as that obtained with the same mutant in replicate experiments. With these levels of supplementation, the mycelium also shows a deep purple pigmentation. As the supplement is increased to 80 or 160 mg of adenine/liter, the amount of pigmentation decreases and the mycelium becomes phenotypically wild type in color. If complementation between *ad-3A* mutants is equivalent to complementation between *ad-3A* and *ad-3B* mutants, then in *ad-3A* heterokaryons we would expect a marked increase in growth rate and a decrease in the intensity of pigmentation of the mycelium.

The possibility of complementation between different *ad-3A* mutants was tested by comparing the growth rates of four forced heterokaryons, two heterozygous for a given pair of *ad-3A* mutants and the other two homozygous (e.g., A1 *inos* + A3 *pan* and A3 *inos* + A1 *pan* compared with A3 *inos* + A3 *pan* and A1 *inos* + A1 *pan*). All possible pairwise combinations of the 13 *ad-3A* mutants were tested in duplicate at ~ 30°C. Most of the combinations were tested with supplementation of both 5 and 10 mg of adenine/liter. Representative results of tests made with mutants A1, A2, and A3 over a wider range of supplementation are given in Table 2.

No evidence of complementation was found between any pairwise combination of the 13 *ad-3A* alleles, that would be indicated by either a change in the growth rate expected with a given level of supplementation or by a change in the level of pigmentation of the mycelium. There was no greater variation in the growth rate with a given level of adenine supplementation with forced heterokaryons heterozygous in the *ad-3A* region than with forced heterokaryons homozygous for a given *ad-3A* mutant.

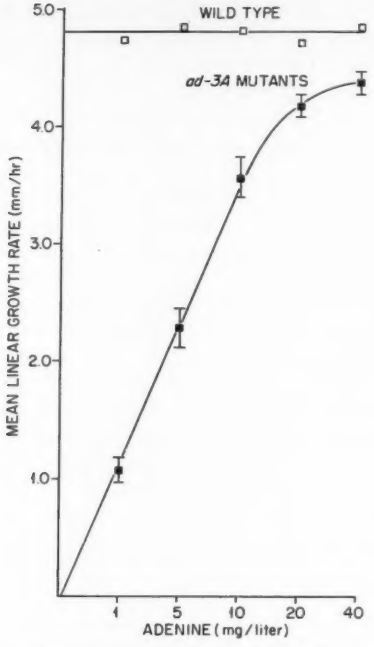


FIGURE 1.—Average linear growth rates of forced heterokaryons homozygous for different *ad-3A* mutants or homozygous for the wild-type allele on minimal medium supplemented with various levels of adenine at 28°–32°C. Vertical lines are 95 percent confidence limits of the mean.

TABLE 2

Linear growth rates of forced heterokaryons heterozygous and homozygous for* ad-3A *mutants A1, A2, and A3 on differentially supplemented media*

Forced heterokaryons	Growth rate with various levels of adenine (mg/liter)				
	1	5	10	20	40
inos + *pan*	0.39	0.39	0.44	0.45	0.45
A1 *inos* + A1 *pan*	0.17	0.24	0.38	0.42	0.42
A2 *inos* + A2 *pan*	0.18	0.27	0.40	0.43	0.45
A3 *inos* + A3 *pan*	0.16	0.26	0.37	0.43	0.41
A1 *inos* + A2 *pan*	0.15	0.28	0.40	0.43	0.42
A2 *inos* + A1 *pan*	0.15	0.26	0.40	0.44	0.44
A1 *inos* + A3 *pan*	0.15	0.23	0.40	0.41	0.42
A3 *inos* + A1 *pan*	0.16	0.25	0.40	0.40	0.42
A2 *inos* + A3 *pan*	0.13	0.19	0.35	0.43	0.46
A3 *inos* + A2 *pan*	0.15	0.26	0.35	0.42	0.44

* Rate: centimeters per hour during a 96-hr period at 28°–32°C.

Analysis of ad-3A *crosses*

Another method of detecting interallelic complementation is by formation of PWT's (PITTENGER 1954) in allelic crosses. Inclusion of different allelic mutants in the same nucleus by nondisjunction of homologous chromosomes and the eventual breakdown of the disomic into a heterokaryon has been a particularly useful and sensitive test for complementation in the analysis of *pyr-3* mutants (MITCHELL and MITCHELL 1956) and the *am* locus (PATEMAN and FINCHAM 1958). CASE (personal communication) found in the analysis of *pan-2* mutants that the PWT test is a more sensitive test for complementation than the heterokaryon test, especially in detecting complementation between mutants very closely linked on a complementation map. This is especially significant with the *pan-2* system, since all of the mutants were induced in wild-type strain 74A or essentially isogenic derivatives, and it does not seem that the difference in sensitivity can be attributed to small differences in genetic background that might affect heterokaryon formation and maintenance.

Crosses with a marked strain of mutant ad-3A*(2):* Various difficulties in the analysis of interallelic crosses of *ad-3A* or *ad-3B* mutants have been discussed previously (DE SERRES 1956). In brief, whereas *ad-3A* × *ad-3B* crosses are fully fertile, interallelic crosses produce very few perithecia and ascospores. In addition, about 90 percent of the ascospores are sterile and even with a number of crosses of the same type, the large populations of viable ascospores necessary for an adequate analysis of such crosses can be obtained only with considerable difficulty.

Because of these crossing problems, the analysis was focused on crosses of the 13 *ad-3A* mutants to a marked strain of mutant A2 (a *hist-2 ad-3A*(2) *nic-2*). The presence of closely linked adjacent markers (*hist-2* about two units to the left and *nic-2* about three units to the right) permits identification of individual components of any PWT's formed as well as an ordering of individual *ad-3A* mutants, with reference to the markers and mutant A2, if any wild-type progeny are recovered. All crosses were made on liquid synthetic cross medium (WESTERGAARD and MITCHELL 1947) supplemented with 1.0 mg of adenine, 0.1 mg of histidine, and 0.01 mg of niacin per milliliter and two percent sucrose in test tubes with filter paper partitions.

All crosses were analyzed by the overlayering technique of NEWMEYER (1954). It was not possible to include all mutants in this analysis, however, since crosses involving mutants A3 and A9 were sterile, no mature perithecia were formed with those of A3, and there were numerous perithecia but no ascospores in those of A9. Also too few ascospores were formed in the crosses of A6 and A7 and selfings of all mutants for plating analysis. Analysis of the selfing of A2 was made possible only by pooling the contents of a very large number of cross tubes.

Since the majority of ascospores in such crosses are not ejected from the perithecia, they were collected in the following manner: The entire contents of the cross tubes were macerated with a spatula in distilled water, and the macerate was filtered through a series of stainless steel sieves or several layers of cheese-

cloth to remove the coarse debris. Ascospores were then freed from conidia and the remaining fine debris by repeated centrifugation, decanting, and washing. Such a method vastly increased the quantity of ascospores available from a given cross with little or no change in viability.

Aliquots of ascospores of known concentration suspended in a viscous 0.15 percent agar solution were plated both (1) in minimal medium supplemented with histidine, adenine, and niacin to estimate the total viable population and (2) in minimal medium supplemented with histidine and niacin to recover adenine-independent progeny. All adenine-independent progeny were subcultured, tested on differentially supplemented media to determine genotype with reference to the adjacent markers, and then crossed to the appropriate mating type of the marked strain *hist-2 ad-3A*(2) *ad-3B*(35203) *nic-2* (GILES, DE SERRES, and BARBOUR 1957) to distinguish between true and pseudowild-type progeny. Pseudowild types are readily distinguished in the analysis of such crosses since they yield 100 percent adenine-requiring progeny, whereas true wild types give only 50 percent such progeny. In addition, a sample of the adenine-independent progeny from crosses of true wild types was tested to determine if their genotypes were the same as the adenine-independent parental type. Such tests detect pseudowild type progeny resulting from nondisjunction of adenine-independent recombinants and a parental chromosome.

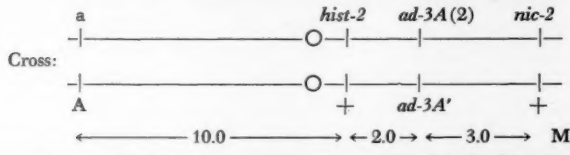
Since *ad-3A* + *ad-3B* combinations show complete complementation, estimates of the frequency of pseudowild type progeny expected in these crosses were obtained from crosses of various *ad-3B* mutants to the same *hist-2 ad-3A*(2) *nic-2* strain (stock culture 74A-YU192-1a) used in the present analysis. Although the analysis of such *ad-3A* × *ad-3B* crosses (DE SERRES unpublished) has shown that crosses of an occasional mutant may have a markedly higher frequency of pseudowild-type progeny than other essentially isogenic allelic mutants, the average expected frequency of such progeny for *ad-3B* mutants induced in a 74A background is one per 16,339 viable progeny and for *ad-3B* mutants of different origin one per 5,256.

The numbers of adenine-independent progeny of both types recovered from individual crosses are given in Table 3. No adenine-independent progeny were recovered from the selfing of mutant A2, but there were a number of adenine-independent progeny in all the other crosses. The frequency of true wild-type progeny show that all mutants are closely linked to A2. However, the marked lack of asymmetry in the numbers of true wild type progeny of crossover genotype (H++ or ++N) makes it impossible to order the mutants with reference to A2. The pattern of marker segregation among the true wild-type progeny in these crosses is quite similar to that obtained by CASE and GILES (1958b) with very closely linked mutants in group B5 of the *pan-2* locus that give a similar frequency of *pan* phototrophs in intercrosses.

Whereas adenine-independent progeny were recovered from all the intercrosses, pseudowild types were found among the progeny of only two crosses. However, neither of the pseudowild type isolates resulted from interallelic complementation between different *ad-3A* mutants. One from the cross A8 × A2,

TABLE 3

Frequency and genotypes of adenine-independent isolates obtained from crosses of ad-3A *mutants with a marked strain of* ad-3A *mutant A2*



Strain	Total viable ascospores × 10⁴*	Adenine-independent progeny							
		Pseudowild types		True wild types					
		Expected	Found	No.	Frequency	Genotype			
						H++	++N	H+N	+++
A1	19.4	11.9	0	68	0.035	13	17	12	26
A2	4.8	. . .		0	. . . .				
A4	68.1	41.7	0	104	0.015	17	18	34	35
A5	84.4	51.7	1†	35	0.004	2	5	12	16
A8	107.4	65.7	1†	109	0.010	11	13	32	53
A10	4.7	2.9	0	16	0.034	1	1	7	7
38701	5.2	9.8	0	18	0.035	4	2	4	8
38709	3.0	5.7	0	9	0.029	3	3	3	0
68306	6.9	13.0	0	17	0.025	4	6	4	3

* Pooled data from several experiments on the same cross.
† See text for discussion.

which tested originally as + + + with reference to markers, was actually a disomic resulting from nondisjunction of a niacin-requiring recombinant with an A8 parental chromosome. The other pseudowild type, also + + +, was recovered from the cross A5 × A2. In this case, nondisjunction involved a nonrequiring recombinant (+ + +) and an A5 parental chromosome. The + + + chromosome carried a new morphological mutation, presumably of spontaneous origin, which greatly restricted the ultimate size of colonies formed by conidia homokaryotic for the mutation. The recovery of these rare PWT's between recombinant and parental chromosomes among the adenine-independent progeny from these crosses indicates that PWT's involving different *ad-3A* mutants with mutant A2 must have also occurred at the higher frequencies expected. Thus the failure to recover PWT progeny involving different *ad-3A* mutants may be interpreted as a failure to obtain interallelic complementation in any of the combinations tested in this manner.

DISCUSSION

The present experiments with 13 *ad-3A* mutants, although they provide no evidence for interallelic complementation at this locus, do give some answers to the question of the significance of the negative results obtained in heterokaryon tests with genetically unmarked *ad-3* strains. Since essentially all the mutants used in the analysis of the *ad-3* region were induced in the same wild-type strain,

they have the same genes controlling heterokaryon formation and maintenance and should be fully compatible (GARNJOBST and WILSON 1956). In none of the present experiments with forced heterokaryons of *ad-3A* mutants nor in previous experiments with various *ad-3A* + *ad-3B* combinations (DE SERRES 1956) has there been any indication that *ad-3* mutants do not form fully compatible heterokaryons. Thus we have the reassurance that, when conidial mixtures of different genetically unmarked *ad-3* mutants are made on minimal medium, a negative reaction indicates that the mutants are not capable of complementation.

Studies of groups of allelic mutants of diverse origin have shown that interallelic complementation is widespread in Neurospora (see CATCHESIDE and OVERTON 1958). There is no substantial body of data as yet on complementation analyses on primary (produced in the original wild-type strain) mutants of spontaneous origin, but five out of seven secondary (produced in a wild-type revertant) *ad-4* mutants of spontaneous origin are capable of complementation (WOODWARD, PARTRIDGE, and GILES 1958); whereas only 30 percent of the ultraviolet-induced *arg-1* mutants (CATCHESIDE and OVERTON 1958) and 21 percent of the X-ray-induced primary *ad-4* mutants were capable of complementation. Of the *ad-3A* mutants used in the present experiments, A2 and A5 were of spontaneous origin, A1, A3, A4, and A6-A10 were derived from X-irradiated conidia, and mutants 38701, 38709, and 68306 were induced by ultraviolet light (BEADLE and TATUM 1945). Thus it seems highly unlikely that the failure to obtain complementation can be attributed to the origin of the mutants.

Since various *ad-3A* intercrosses with mutant A2 yielded adenine-independent progeny, there seems little doubt that the *ad-3A* region consists of an undetermined number of mutable sites. Failure to obtain interallelic complementation does not necessarily mean that alteration of any one or combination of these sites produces mutants with identical defects. If complementation occurs by dissociation and reassociation of polypeptide chains of enzyme molecules with nonidentical defects to produce nondefective, functionally active enzyme, then the present analysis shows only that the 13 *ad-3A* mutants have some defect in common. Some of these mutants may be capable of complementation but only with *ad-3A* mutants of a type not included in the present analysis.

The most probable explanation for the failure to obtain allelic complementation among the present group of *ad-3A* mutants is the somewhat limited sample size. Although it is possible that complementation cannot occur among *ad-3A* mutants, this point has certainly not been proved in the present analysis. Mutations that occur at points within the *ad-3A* region that might permit complementation could be relatively rare events in themselves or even show a mutagen specificity. We hope to get more conclusive evidence of complementation in the *ad-3* region by increasing the sample size to a couple of hundred *ad-3A* and *ad-3B* mutants and using mutants of different mutagenic origin. This approach has been made feasible by the development of a forward-mutation technique (DE SERRES and KØLMARK 1958) that permits ready identification and recovery of *ad-3* mutants in treated populations of conidia.

SUMMARY

(1) Heterokaryon tests on a series of 13 genetically unmarked *ad-3A* strains of independent origin showed no evidence for interallelic complementation in any pairwise combination.

(2) The possibility that such negative results might be inconclusive was investigated with forced heterokaryon tests on genetically marked strains.

(3) No evidence for interallelic complementation in any pairwise combination of these mutants was found in forced heterokaryons grown on unsupplemented medium or medium supplemented with suboptimal levels of adenine.

(4) The recovery of true wild type progeny from crosses of different *ad-3A* mutants with a marked strain of mutant A2 suggest that the *ad-3A* region consists of an undetermined number of mutable sites, but the lack of asymmetry in marker segregation among the adenine-independent progeny did not permit an ordering of the mutants with reference to mutant A2 and the adjacent markers *hist-2* and *nic-2*.

(5) The failure to recover pseudowild type progeny involving both *ad-3A* parental chromosomes from the fertile intercrosses provides further evidence for the absence of interallelic complementation in these combinations.

(6) The present experiments firmly substantiate the original interpretation of the negative heterokaryon tests with genetically unmarked but isogenic *ad-3* mutants: that, under the experimental conditions used, such results are significant and provide evidence for the absence of complementation.

(7) The present data are consistent with the *ad-3A* locus being a one-cistron locus with an undetermined number of recombinable, mutable sites.

LITERATURE CITED

BARRATT, R. W., D. NEWMEYER, D. D. PERKINS, and L. GARNJOBST, 1954 Map construction in *Neurospora crassa*. Advances in Genet. **6**: 1–93.

BEADLE, G. W., and E. L. TATUM, 1945 Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Botany **32**: 678–686.

CASE, M. E., and N. H. GILES, 1958a Evidence for tetrad analysis for both normal and aberrant recombination between allelic mutants in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **44**: 378–390.

1958b Recombination mechanisms at the *pan-2* locus in *Neurospora crassa*. Cold Spring Harbor Symposia Quant. Biol. **23**: 119–135.

CATCHESIDE, D. G., and A. OVERTON, 1958 Complementation between alleles in heterokaryons. Cold Spring Harbor Symposia Quant. Biol. **23**: 137–140.

DE SERRES, F. J., 1956 Studies with purple adenine mutants in *Neurospora crassa*. I. Structural and functional complexity in the *ad-3* region. Genetics **41**: 668–676.

1958 Studies with purple adenine mutants in *Neurospora crassa*. III. Reversion of X-ray-induced mutants. Genetics **43**: 187–206.

DE SERRES, F. J., and H. G. KØLMARK, 1958 A direct method for determination of forward-mutation rates in *Neurospora crassa*. Nature **182**: 1249–1250.

FINCHAM, J. R. S., and J. A. PATEMAN, 1957 Formation of an enzyme through complementary action of mutant alleles in separate nuclei in a heterokaryon. Nature **179**: 741–742.

GARNJOBST, L., 1955 Further analysis of genetic control of heterokaryosis in *Neurospora crassa*. Am. J. Botany **42**: 444–448.

GARNJOBST, L., and J. F. WILSON, 1956 Heterokaryosis and protoplasmic incompatibility in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **42**: 613–618.

GILES, N. H., F. J. DE SERRES, and E. BARBOUR, 1957 Studies with purple adenine mutants in *Neurospora crassa*. II. Tetrad analysis from a cross of an *ad-3A* mutant with an *ad-3B* mutant. Genetics **42**: 608–617.

GILES, N. H., C. W. H. PARTRIDGE, and N. J. NELSON, 1957 The genetic control of adenylosuccinase in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **43**: 305–317.

HUEBSCHMAN, C., 1952 A method for varying the average number of nuclei in the conidia of *Neurospora crassa*. Mycologia **44**: 599–604.

LACY, A. M., 1959 A genetic analysis of the *Td* locus in *Neurospora crassa*. Ph.D. Thesis, Yale University, New Haven, Conn.

LACY, A. M., and D. M. BONNER, 1958 Complementarity between alleles at the *Td* locus in *Neurospora crassa*. Proc. 10th Intern. Congr. Genet. **2**: 157.

MITCHELL, M. B., and H. K. MITCHELL, 1956 Tests for nonallelism at the pyrimidine-3 locus of Neurospora. Genetics **41**: 319–326.

NEWMEYER, D., 1954 A plating method for genetic analysis in Neurospora. Genetics **39**: 604–618.

PATEMAN, J. A., and J. R. S. FINCHAM, 1958 Gene-enzyme relationships at the *am* locus in *Neurospora crassa*. Heredity **12**: 317–332.

PITTENGER, T. H., 1954 The general incidence of pseudo-wild types in *Neurospora crassa*. Genetics **39**: 326–342.

PITTENGER, T. H., and K. C. ATWOOD, 1956 Stability of nuclear proportions during growth of Neurospora heterokaryons. Genetics **41**: 227–241.

WESTERGAARD, M., and H. K. MITCHELL, 1947 Neurospora. V. A synthetic medium favoring sexual reproduction. Am. J. Botany **34**: 573–578.

WOODWARD, D. O., C. W. H. PARTRIDGE, and N. H. GILES, 1958 Complementation at the *ad-4* locus in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **44**: 1237–1244.

THE GENETICAL ANALYSIS OF CHARACTERS WITH CONTINUOUS VARIABILITY ON A MENDELIAN BASIS. III. DIHYBRID SEGREGATION

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AMONG the tomato F_1 hybrids grown under glass in Hohenheim in 1958, the hybrid of the lines Fr 57 × Oc 57 was of particular interest for showing heterosis in a number of characters, e.g., leaf size, length of shoot, insertion height of inflorescence, number of flowers, earliness, etc.

Segregation by discriminant function of the character cotyledon size in the F_2 of Bonner Best × Professor Rudloff had led to a clear 3:1 segregation ratio (WEBER 1959); therefore, it was obvious that an F_2 analysis of the cotyledons of Fr 57 × Oc 57 should also be made.

METHODS

One hundred and fifty seeds each of the parents Fr 57 (=P_1), Oc 57 (=P_2) and of their F_1, and 1200 seeds of F_2 were sown singly into seed boxes at distances of 3 cm × 3 cm. Five weeks later, one cotyledon of each plant was cut off, pressed and kept in a herbarium. Then the length in mm (=x_1) and the area in cm² × 10 (=x_2) were found of 100 cotyledons taken at random from each of the parents P_1 and P_2 and from F_1. Of the F_2, a total of 1135 cotyledons was measured.

Table 1 contains the frequency distributions of the single measurements x_1, x_2 and of the total measurements X for the parents P_1, P_2 and for F_1. Figures 1a, 1b, 1c show clearly that the distributions of the parents P_1 and P_2 overlap almost completely. The F_1 distributions lie outside those of the parents. The differences between the mean values of F_1 and of the parents are significant. (For the values of x_1, $t = 5.92^{+++}$ and for x_2, $t = 6.43^{+++}$.) As the differences between the mean values of the parents P_1 and P_2 are not significant, the discriminant function $X = b_1x_1 + b_2x_2$ has been made up only of the collectives P_1 and F_1. It was obtained from the following equation:

$$1750\, b_1 + 892\, b_2 = 2.49$$
$$892\, b_1 + 686\, b_2 = 1.70$$

$$b_1 = 0.000473$$
$$b_2 = 0.001862$$
$$\text{or } X = 1x_1 + 3.94x_2$$

misclassification 1/2 P = 0.11

TABLE 1

Frequency distributions of the single measurements x_1, x_2 *and of the total measurement* X *for the parents* P_1 *and* P_2 *and their* F_1

x_1 (see Figure 1a)				x_2 (see Figure 1b)				X (see Figure 1c)			
Class mean	Frequency of: P_1	P_2	F_1	Class mean	Frequency of: P_1	P_2	F_1	Class mean	Frequency of: P_1	P_2	F_1
27,5	0	0	0	8	0	0		57		0	
29,5	1	5	1	9	1	4	0	62	0	1	
31,5	6	5	3	10	10	6	4	67	2	4	0
33,5	29	10	8	11	13	15	4	72	10	9	4
35,5	26	33	14	12	30	20	9	77	11	12	4
37,5	19	25	17	13	19	18	17	82	32	18	10
39,5	14	12	36	14	15	13	24	87	17	17	12
41,5	3	9	13	15	7	11	17	92	12	13	15
43,5	2	1	5	16	3	9	10	97	10	12	27
45,5	0	0	3	17	1	2	8	102	4	10	14
47,5			0	18	1	1	4	107	1	2	5
				19	0	1	3	112	1	1	4
				20		0	0	117	0	1	5
								122		0	0

TABLE 2

Frequency distributions of the single measurements x_1, x_2 *and of the total measurement* X *for* F_2

x_1 (see Figure 1d)				x_2 (see Figure 1e)		X (see Figure 1f)	
Class mean	Frequency	Class mean	Frequency	Class mean	Frequency	Class mean	Frequency
22	0	42	32	5	0	42	0
23	1	43	23	6	2	47	2
24	3	44	9	7	4	52	4
25	5	45	6	8	11	57	11
26	4	46	0	9	42	62	18
27	6	47	1	10	62	67	57
28	16	48	0	11	134	72	69
29	22	49	1	12	161	77	109
30	45	50	1	13	164	82	169
31	54	51	0	14	208	87	145
32	67			15	150	92	166
33	79			16	93	97	175
34	86			17	59	102	101
35	125			18	26	107	59
36	143			19	8	112	23
37	103			20	10	117	15
38	100			21	0	122	10
39	77			22	1	127	1
40	78			23	0	132	1
41	48					137	0

Table 2 shows the frequency distributions of the single measurements x_1, x_2 and of the total measurement X for F_2 with 1135 individuals. The continuity of the distributions of x_1 and x_2 in Figures 1d and 1e indicates no segregation. But the distribution of the total measurement X in Figure 1f shows by its V-shaped notch that two collectives are present. The area on the right of the notch is to that on the left as 8.8:7.2, that is 624:511 individuals. Assuming a 9:7 segregation, then $x^2 = 0.703$ and $P = 0.4$.

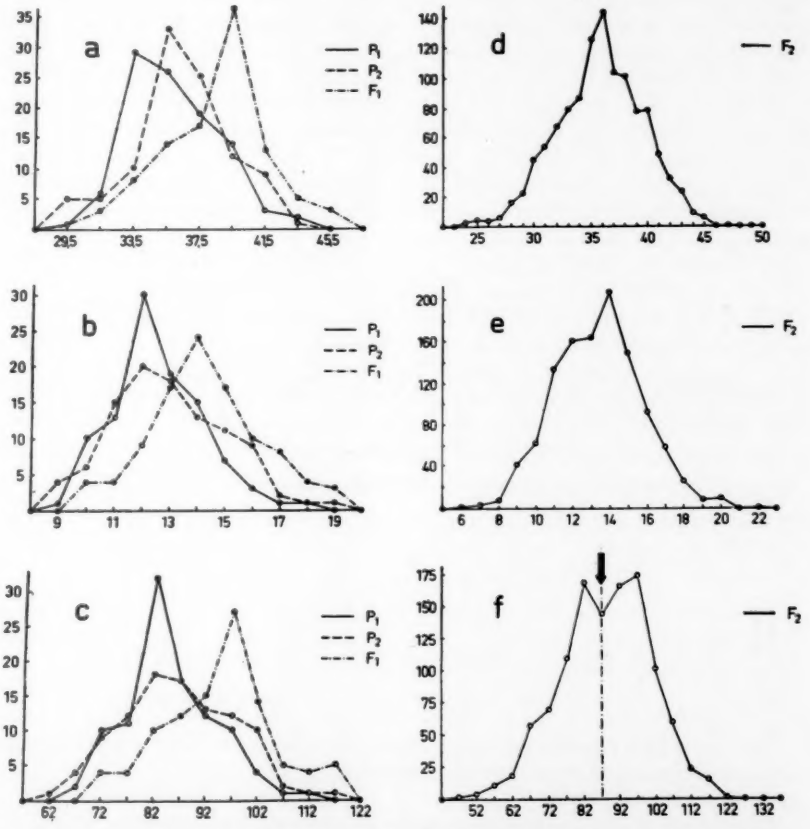

FIGURE 1.—a. Frequency distributions of the single measurement x_1 (length of cotyledon) for the parents and F_1. b. Frequency distributions of the single measurement x_2 (area of cotyledon) for the parents and F_1. c. Frequency distributions of the total measurement X of x_1 and x_2 for the parents and F_1. d. Frequency distribution of the single measurement x_1 for F_2. e. Frequency distribution of the single measurement x_2 for F_2. f. Frequency distribution of the total measurement X of x_1 and x_2 for F_2.

Normally, with such a relatively large number of F_2 individuals, a smaller χ^2 would be expected if indeed there is a 9:7 segregation. A 9:7 segregation is assumed where the parents are phenotypically uniform in respect of the character being investigated; therefore, P_1 would be represented by *AAbb*, P_2 by *aaBB* and F_1 by *AaBb*. The F_2 ought to show nine individuals phenotypically uniform with the F_1 and seven phenotypically uniform with the parents. Therefore, an F_2 distribution based on a 9:7 segregation was worked out from the distributions of the total measurements of Figure 1c. As the distributions of the collectives P_1 and P_2 overlap to a large extent, the arithmetical mean of their frequencies for the respective classes was multiplied by seven and the frequency of F_1 by nine. (See Figure 2.) By adding together the distributions (1) and (2) in Figure 2, the estimated F_2 distribution (3) is obtained. This coincides largely with the empirical F_2 distribution (4). In the estimated distribution there is, however, no distinct V-shaped notch through which a border line might be drawn; instead there are two almost equal-sized ordinates surrounded by two taller ones leading to a roughly U-shaped depression so that the border line ($=K_2$) is to be drawn through the middle of this U. In Figure 2, the area below the distribution curve (3) and on the right of the border line K_2, is to that on the left of K_2 as 8.7:7.3, although the areas below the distributions (2) and (1) are as 9:7. The deviation from the 9:7 ratio is caused by the asymetrical distributions of (1) and (2) in Figure 2. The fact that the segregation of 8.8:7.2 in the empirical F_2 distribution (4) is based on a 9:7 segregation, may be concluded from the proximity of the segregation ratio of the estimated F_2 distribution (3) of 8.7:7.3 (617:518 individuals) to the empirical distribution of 8.8:7.2 (624:511 individuals); there is no significant deviation ($\chi^2 = 0.174$; $P = 0.68$). In addition, estimation of the mean value of the total measurement of the F_2 distribution $\overline{X}_{F_2} = 90.90$ based on the mean values of the parents and F_1, largely coincides with the empirical mean value of the F_2, namely $\overline{X}_{F_2} = 88.24$.

$$\frac{1}{16}\left[7 \times \frac{1}{2}\left(\overline{X}_{P_1} + \overline{X}_{P_2}\right) + 9 \times \overline{X}_{F_1}\right] = \overline{X}_{F_2}$$

$$\frac{1}{16}\left[7 \times \frac{1}{2}\left(87.11 + 85.20\right) + 9 \times 94.60\right] = 90.90 \text{ estimated mean}$$

$$88.24 \text{ empirical mean}$$

The above mentioned empirical example shows that even where the frequency peaks are very near to each other, segregation by discriminant function is possible as illustrated by Figure 1.

The theory of a 9:7 segregation in the F_2 generation is supported by an example of backcross analysis.

Segregation in the backcross generation: If the 9:7 segregation obtained from the distribution of the total measurement of the F_2 generation is correct, then the backcross generation ought to segregate in the ratio of 1:1, even if, as in this experiment, the seed used was a mixture of the backcrosses $P_1 \times F_1$ and $P_2 \times F_1$.

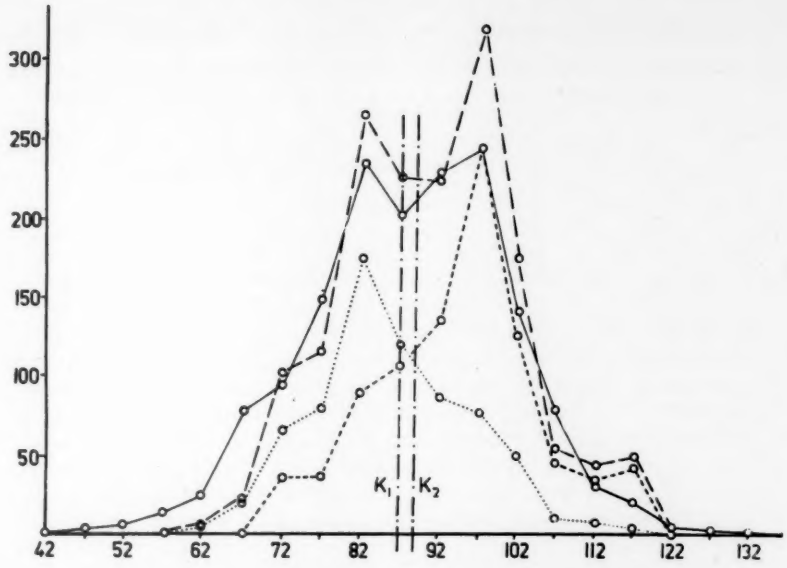

FIGURE 2.—Frequency distributions of the total measurement X for the parents, F_1 and F_2. (Further explanations in the text.)

Table 3 and Figure 3 show the frequency distributions of the single measurements x_1 and x_2 and of the total measurement X for 1248 individuals. Although the presence of more than one collective is to be inferred from the abnormal distributions of the single measurements x_1 and x_2 in Figures 3a and 3b, there is no indication of the actual segregation ratio. The latter may be inferred only from the distribution of the total measurement for the backcross generation—it shows a 1:1 segregation ratio, i.e., 620:628 individuals. ($x^2 = 0.0514$, $P = 0.81$).

The assumption of a 9:7 segregation, which should, however, be corroborated by further empirical results, supports the theory of a number of authors, e.g., MALINOWSKI (1952), JINKS (1956), JINKS and JONES (1958), etc., that the higher performance of F_1 hybrids (i.e., heterosis) is controlled by complementary factors—that is to say interaction of nonallelic genes.

(The extensive tables of measurements may be obtained on application to the author.)

TABLE 3

Frequency distributions of the single measurements x_1, x_2 and of the total measurement X for the backcross generation

x_1 (see Figure 3a)				x_2 (see Figure 3b)		X (see Figure 3c)	
Class mean	Frequency	Class mean	Frequency	Class mean	Frequency	Class mean	Frequency
22	0	40	103	7	0	47	0
23	1	41	40	8	3	52	1
24	1	42	31	9	20	57	2
25	1	43	11	10	34	62	4
26	0	44	10	11	142	67	28
27	0	45	2	12	155	72	81
28	2	46	1	13	250	77	107
29	23	47	0	14	210	82	221
30	28			15	206	87	176
31	45			16	132	92	174
32	99			17	74	97	226
33	112			18	12	102	122
34	124			19	7	107	71
35	146			20	3	112	22
36	120			21	0	117	8
37	122					122	4
38	118					127	1
39	108					132	0

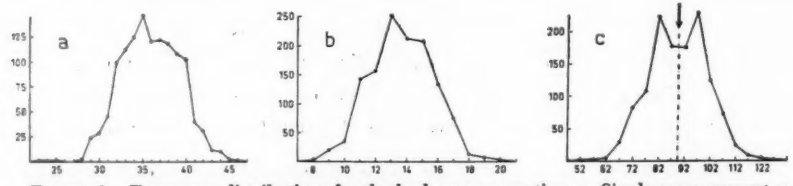

FIGURE 3.—Frequency distributions for the backcross generation: a. Single measurement x_1; b. Single measurement x_2; c. Total measurement $X = f(x_1) + f(x_2)$; 1:1 segregation.

SUMMARY

The method described in WEBER (1959) for genetical analysis of characters with continuous variability on a Mendelian basis by means of discriminant function, was applied to an F_2 and a backcross generation. The inheritance of cotyledon size in two lines of tomato (*Lycopersicum esculentum* Mill.) was found to be controlled by two factors.

LITERATURE CITED

JINKS, J. L., 1956 The F_2 and backcross generation from a set of diallel crosses. Heredity **10**: 1–30.

JINKS, J. L., and R. M. JONES, 1958 Estimation of the components of heterosis. Genetics **43**: 223–234.

MALINOWSKI, E., 1952 The problem of heterosis. VI. Different shapes of the F_2 frequency distributions. Bull. Intern. Acad. Polon. Classe Sci. math. nat. Sér. B. 1/3: 41–76.

WEBER, E., 1957 Betrachtungen zur Diskriminanzanalyse. Z. Pflanzenzücht. **38**: 1–36.

1959 The genetical analysis of characters with continuous variability on a Mendelian basis. I. Monohybrid segregation. Genetics **44**: 1131–1139.

HETEROSIS IN DDT RESISTANT AND SUSCEPTIBLE POPULATIONS OF DROSOPHILA MELANOGASTER

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THE phenomenon of heterosis has generally been studied in relation to some arbitrarily selected trait such as size, weight, or yield. Heterosis is then regarded as being manifested by an increase in the chosen trait. Although various definitions have been proposed (GOWEN 1952), heterosis is most commonly and most meaningfully defined as an increase in the chosen trait in crossbred individuals as compared to the better of their more inbred parents. While heterosis is usually considered to affect all of the characters of hybrids, it is actually only measured in relation to the trait or traits selected for study. Recently DOBZHANSKY (in GOWEN 1952) has equated heterosis with adaptive value and has called increased size or vigor "luxuriance" when it does not contribute to fitness. This usage, while it has the advantage of referring always to the same trait, adaptive value, may lead to considerable confusion since it is entirely possible for smaller size, for example, to confer greater adaptive value than large, and hence a direct contradiction between the historical meaning of the word (SHULL 1948) and this new definition will result. The experiments to be reported were designed to test some of the concepts which have grown up around the phenomenon of heterosis.

METHODS

The flies used in the experiments were drawn from the two most resistant stocks (731 R and 91 R) among several developed during six years of exposure to DDT. These populations had been maintained in "population bottles" (REED and REED 1948) in which were placed slips of filter paper impregnated with DDT. In this way all stages of the life cycle were likely to be exposed. Separate unexposed controls (731 C and 91 C) were run for each population. At the time of the crosses each of these four stocks had formed an isolated breeding population for a period of six years. These strains were both descended from flies captured in wild populations of *Drosophila melanogaster* in St. Paul, Minnesota, in the summer of 1952. Large numbers of flies rather than single inseminated females were collected in an effort to have initial populations with considerable genetic variability. (MERRELL and UNDERHILL 1956, gave the size of the original populations as up to 50 flies, but a check of the notes made at the time of collection indicates that for these two populations it was more like a few hundred.) Their heterozygosity was indicated by the continued increase in resistance of the exposed populations. This increase was evidenced by the need to increase the dose to the populations from 0.5 mg to 40 mg during this period as well as by the separate tests run to deter-

mine the ED_{50}. For further details on the methods used and the results with inbred and laboratory stocks see MERRELL and UNDERHILL 1956.

The following types of flies were tested:

	Control	Resistant
P_1	731 C	731 R
	91 C	91 R
F_1	731 C ♀♀ × 91 C ♂♂	731 R ♀♀ × 91 R ♂♂
	91 C ♀♀ × 731 C ♂♂	91 R ♀♀ × 731 R ♂♂
F_2	731 C ♀♀ × 91 C ♂♂	731 R ♀♀ × 91 R ♂♂
	91 C ♀♀ × 731 C ♂♂	91 R ♀♀ × 731 R ♂♂

The traits which were measured were DDT resistance, fecundity, and fertility. All of the tests for a given generation were run concurrently, and all of the generations were run in a little over a month in a constantly lighted room held at 21 ± 1°C and 50 percent relative humidity so that comparisons within and between generations should be reasonably valid.

The flies for testing and for making the crosses were derived from four half-pint culture bottles for each original population. Each of the four bottles contained as parents 20 females and 20 males taken from the population bottles. No effort was made to get virgin females so that the flies in each bottle were descended from at least 40 parents and possibly quite a few more. Hence, the flies from each population used in the experiments came from at least $4 \times 40 = 160$ parents and probably more. In this way it was hoped that a broad sample of the gene pool of each population would be obtained. The progeny from these four cultures were collected twice daily and were mixed before flies were taken for use in the crosses or in the various tests.

Reciprocal crosses were made between 731 C and 91 C and between 731 R and 91 R, four bottles for each cross, with 20 virgin females and 20 males per bottle, again a total of 160 parents for each F_1 population. The four F_1 bottles for a particular cross (e.g., 91 C × 731 C) served as the source of flies for the tests and of the 160 flies which served as parents of the 91 C × 731 C F_2 generation.

For the tests of DDT resistance, only females were used (See MERRELL and UNDERHILL 1956 for further details). The test period had to be extended to 24 hours from the six hours previously used in order to kill enough flies of the resistant stocks to get an estimate of the ED_{50}.

For the fecundity tests eight to ten females and twice as many males were placed in a fresh bottle of food for three days. On the fourth day five of these females were placed individually in creamers containing two males apiece and food darkened with India ink. The flies were transferred every 24 hours to a fresh creamer to get the egg production on the fourth, fifth, and sixth day after eclosion. These 72-hour tests were run for 30 females of each stock, with five females of each of the four stocks being started each day for six consecutive days. In order to test fertility these cultures were set aside for 15 days at which time the adults were counted. A second count was made after two more days as a check for late

emerging flies, and the creamers were then discarded. The possibility of a negative correlation between fecundity and fertility was checked because of the possibility that the food in creamers containing large numbers of larvae might be exhausted and thus a false picture of fertility be presented. However, a plot of fertility against fecundity indicated no such correlation. Fertility, of course, was influenced by the genotypes of the offspring as well as those of both parents and was, therefore, a more complex trait than either fecundity or DDT resistance which, as measured here, were essentially traits only of the females.

RESULTS

Tables 1 and 2 show the results of the tests for DDT resistance in the resistant and control populations and in their F_1 and F_2 hybrids. The ED_{50}'s in Table 2 were estimated by the SPEARMAN-KÄRBER method (See FINNEY 1952; KEMPTHORNE, BANCROFT, GOWEN, and LUSH 1954). As a check, probit analyses by the arithmetical method (FINNEY 1952) were also calculated for the resistant stocks. Three of the six probit regression lines showed a significant χ^2 tests for hetero-

TABLE 1

DDT resistance of control and resistant populations and F_1 and F_2 hybrids (24 hr test)

	Control					
	P_1		F_1		F_2	
	731 C	91 C	731 C × 91 C	91 C × 731 C	731 C × 91 C	91 C × 731 C
N*	125	125	125	125	123	113
Conc DDT (mg)	Percent down†	Percent down	Percent down	Percent down	Percent down	Percent down
100.0	100.0	100.0	100.0	100.0	100.0	100.0
10.0	100.0	100.0	100.0	100.0	99.2	100.0
1.0	100.0	97.6	100.0	100.0	98.4	99.1
0.1	89.6	32.8	30.4	31.2	52.0	64.6
0.01	6.4	0.0	8.0	4.8	7.3	6.2
	Resistant					
	P_1		F_1		F_2	
	731 R	91 R	731 R × 91 R	91 R × 731 R	731 R × 91 R	91 R × 731 R
N*	125	125	125	125	127	137
Conc DDT (mg)	Percent down	Percent down	Percent down	Percent down	Percent down	Percent down
100.0	84.8	68.8	68.0	68.8	78.0	85.4
10.0	67.2	52.8	42.4	48.0	57.5	53.3
1.0	27.2	17.6	5.6	4.8	22.8	28.5
0.1	7.2	1.6	4.8	4.8	9.4	8.0
0.01	1.6	2.5‡	1.6	2.4	1.6	3.6

* Number at each concentration.
† Includes all flies unable to stand, both knockdown and kills.
‡ N = 120.

TABLE 2

ED_{50} for resistant and control populations and F_1 and F_2 hybrids

	Population	Log ED_{50} (Hundredths of mg)		ED_{50} (mg)	Relative resistance
		Spearman-Karber	Arith. probit analysis		
P_1	731 C	1.54 ± .04	.....	0.04	1.
	91 C	2.20 ± .04	.....	0.16	4.
	731 R	3.62 ± .07	3.67 ± .08	4.2	105.
	91 R	4.07 ± .07	4.18 ± .09	11.7	293.
F_1	731 C × 91 C	2.12 ± .04	.....	0.13	3.2
	91 C × 731 C	2.14 ± .05	.....	0.14	3.5
	731 R × 91 R	4.28 ± .07	4.41 ± .10	18.9	473.
	91 R × 731 R	4.21 ± .07	4.34 ± .10	16.3	407.
F_2	731 C × 91 C	1.93 ± .06	.....	0.08	2.0
	91 C × 731 C	1.80 ± .05	.....	0.06	1.5
	731 R × 91 R	3.81 ± .07	3.88 ± .09	6.4	160.
	91 R × 731 R	3.71 ± .07	3.76 ± .08	5.2	130.

geneity, but in each case they were due to a large contribution from classes with small expectations. The log ED_{50}'s obtained by the two methods were in good agreement. Most of the control data, however, was not well suited for probit analysis. While the Spearman-Kärber estimates may lack some precision, the essential features of the data are such that these estimates are quite adequate.

There was no heterosis for resistance in the F_1 controls while in the F_2 the ED_{50} was lower than in the F_1, but not lower than that of the lower parent. The survival of a few F_2 flies at the higher concentrations may indicate that segregation and recombination had produced a few individuals with increased resistance.

The resistant F_1 showed marked heterosis for resistance. The F_2 showed a significant decrease in resistance as compared to the F_1, but fell between the original parental populations in resistance. There is no significant difference between the results from reciprocal crosses in either the control or resistant crosses, except possibly in the control F_2 where $P = 0.05$.

The results of the tests for fecundity are shown in Table 3. It is noteworthy that every female tested laid at least some eggs so that the large variance is due to the variation among females in the number of eggs they produced rather than to some females who laid no eggs at all. Also noteworthy is the low fecundity of the 91 R population, significantly lower than that of 91 C, 731 C, and 731 R, which did not differ significantly from each other.

Significant heterosis for fecundity was observed in the F_1 in both resistant and control hybrids. Surprisingly, in the F_2 only one out of the four populations declined significantly below the fecundity of the F_1 from which it was derived.

The results on fertility (Table 4) are not particularly striking. However, again it is noteworthy that only one female, which laid 133 eggs, failed to produce adult

offspring. The most unusual finding was the low fertility (70.8 percent) of the 731 C females. This reduced fertility was not due to low fertility in just a few females, for only one 731 C female had fertility lower than 50 percent. Furthermore, crowding in the creamers could hardly have been a factor, for the 91 C females, which were tested concurrently in the same batch of creamers, did not differ significantly from the high fecundity of the 731 C females, yet had a high fertility (90.6 percent).

Table 5 summarizes the data for purposes of comparison. The original control populations differed significantly in both resistance and fertility. The control F_1 showed heterosis only for fecundity, not for resistance or fertility. The resistant populations differed significantly from each other in resistance and in fecundity and were approximately 75 and 100 times more resistant than their respective

TABLE 3

Fecundity

Controls			Resistant		
	N females	Eggs/female/72 hr		N females	Eggs/female/72 hr
P			P		
731 C	30	145.3 ± 9.6	731 R	29	138.8 ± 7.5
91 C	30	143.7 ± 7.3	91 R	29	107.2 ± 8.4
F_1			F_1		
731 C × 91 C	30	172.7 ± 8.8	731 R × 91 R	30	163.8 ± 7.4
91 C × 731 C	30	180.4 ± 6.4	91 R × 731 R	30	150.5 ± 6.6
F_2			F_2		
731 C × 91 C	30	174.8 ± 8.6	731 R × 91 R	30	146.4 ± 8.6
91 C × 731 C	30	180.6 ± 9.0	91 R × 731 R	30	145.8 ± 6.5

TABLE 4

Fertility as adults/eggs laid

Controls			Resistant		
	Total eggs	Percent fertility		Total eggs	Percent fertility
P			P		
731 C	4,360	70.8	731 R	4,027	82.0
91 C	3,907	90.6	91 R	3,109	82.8
F_1			F_1		
731 C × 91 C	5,182	87.4	731 R × 91 R	4,916	92.9
91 C × 731 C	5,412	87.9	91 R × 731 R	4,526	90.3
F_2			F_2		
731 C × 91 C	5,245	83.1	731 R × 91 R	4,391	90.9
91 C × 731 C	5,417	91.7	91 R × 731 R	4,374	86.6
Total	29,523			25,343	

TABLE 5

Comparison with respect to DDT resistance, fecundity, and fertility

Control				Resistant			
	Resistance ED_{50} (mg)	Fecundity (eggs/ female/ 72 hr)	Fertility percent		Resistance ED_{50} (mg)	Fecundity (eggs/ female/ 72 hr)	Fertility percent
P				P			
731 C	.04	145.3	70.8	731 R	4.2	138.8	82.0
91 C	.16	143.7	90.6	91 R	11.7	107.2	82.8
F_1				F_1			
731 C × 91 C	.13	172.7	87.4	731 R × 91 R	18.9	163.8	92.9
91 C × 731 C	.14	180.4	87.9	91 R × 731 R	16.3	150.5	90.3
F_2				F_2			
731 C × 91 C	.08	174.8	83.1	731 R × 91 R	6.4	146.4	90.9
91 C × 731 C	.06	180.6	91.7	91 R × 731 R	5.2	145.8	86.6

controls. The resistant F_1 was heterotic for all three traits. While some decreases occurred in the F_2's, in no case was any value found which was lower than that of the lower original parent. While both resistant and control crosses showed heterosis for fecundity, the fecundity of the controls was generally higher than that of the resistant populations.

DISCUSSION

Although only two gene pools were present in duplicate in 1952, after six years of isolation and selection, four distinct populations had evolved. Each differed significantly from each of the other three populations in at least two of the three traits tested. No obvious relationship among these traits is apparent, for the most resistant population (91 R) had the lowest fecundity, the most fecund (731 C) had the lowest fertility, and the most fertile (91 C) was not resistant to DDT.

The most striking heterosis is found in the fecundity of the F_1 controls and in the DDT resistance of the resistant F_1. These results suggest a relationship between previous selection pressures and the degree of heterosis observed. The constant heavy selection pressure exerted by DDT in the resistant populations was absent in the control populations, and only in the F_1 resistant hybrids was heterosis for resistance observed. Similarly, while fecundity must have been important in both types of populations, it can be assumed to have been of relatively greater importance in the absence of DDT so that the higher fecundity and greater degree of heterosis for fecundity in the control populations is perhaps not surprising.

Theories to account for heterosis have been of two major types, which may, for convenience, be termed dominance (Jones 1917) and overdominance (East 1936; Hull 1945). Heterosis due to overdominance is postulated to stem from heterozygosity *per se*. The greater the number of heterozygous loci, therefore, the greater the heterosis expected. Under a strict interpretation of this theory, only the interactions within loci are significant as causes of heterosis.

Under the dominance theory, the genes favorable to increased size or vigor are assumed to be dominant or at least partially dominant. The hybrid will have a greater number of loci with at least one favorable dominant than either parent. Therefore, the minimum number of loci required for the expression of this type of heterosis is two. This type of heterosis may be due simply to the masking of deleterious recessive genes, but it may also be due to nonallelic interactions among the favorable genes. Theoretically, homozygotes equal to or even superior to the heterozygotes should be obtainable, but with many loci involved, the problem of obtaining homozygosity for only the favorable genes becomes extremely complex.

While these theories are often regarded as alternatives, they are by no means mutually exclusive, and the heterosis observed in any particular case may well be due to the masking of deleterious recessives and to favorable interactions, both nonallelic and allelic. It is, moreover, entirely conceivable that in different instances of heterosis, the relative importance of these different factors may vary widely. Furthermore, while the theories are clear-cut, the experimental determination of which mechanism is more important in a given situation is not a simple matter, for ideally it requires a knowledge of the behavior of genes at individual loci in a polygenic system.

In these experiments the data indicate that a finding of heterosis in one trait does not necessarily mean that all traits of the same organism will also manifest heterosis. Here, then, heterosis appears not to be some sort of generalized phenomenon resulting simply from heterozygosity, but must be determined by the genetic situation at the loci which determine the trait or traits under study.

Recent work with natural populations has led to the development of the theory of coadaptation or the integration of the genotype. Natural selection is thought to operate within a breeding population to build up "harmoniously integrated genetic systems." Three levels of integration have been postulated (WALLACE and VETUKHIV 1955): "(1) Integration based upon epistatic interactions between homozygous loci. (2) Coadaptation of different gene arrangements within local populations involving both heterosis and epistasis, and (3) the integration of entire gene pools through selection for heterozygosity." These statements indicate the significance of heterosis to the theory of coadaptation and would seem to imply that overdominance is the mechanism considered to be responsible for the heterosis at the third level (WALLACE 1958).

The considerable and continued increase in resistance in the exposed populations is evidence for the genetic variability of the original populations since it has already been demonstrated (MERRELL and UNDERHILL 1956) that selection for DDT resistance is ineffective in highly inbred stocks and even, in six of seven tested, in several well-known "wild" stocks of *D. melanogaster* which have been maintained as laboratory stocks for many years, and are also presumably quite inbred and relatively homozygous. Therefore, since each population has been maintained as a separate breeding unit consisting of a few hundred individuals confined for more than six years in a very small space, it would seem that conditions were very favorable for the development of coadaptation. However, the

results of the crosses indicate that such integration of the genotypes has not taken place within these populations. The maximum DDT resistance was found in F_1 resistant hybrids and the maximum fecundity in the F_1 control hybrids. In other words, the maxima were found where there should have been minimum coadaptation. This result is frequently reported in the work on coadaptation and would seem to be its most serious weakness. For all three levels of integration discussed by WALLACE and VETUKHIV in 1955, the F_1 hybrids from crosses between individuals from two different breeding populations representing two different integrated gene pools would be expected to be less viable than the parents. The fact that they generally are heterotic requires that some explanation other than coadaptation be invoked to explain this heterosis. Therefore, the heterosis of F_1 interpopulation hybrids represents a serious flaw in the whole argument for coadaptation. Furthermore, since none of the F_2's fell below its lower parent in DDT resistance, fecundity, or fertility, there was no evidence of "hybrid breakdown" in the F_2 in these experiments.

Since coadaptation does not seem to give a satisfactory interpretation for the data, other possibilities may be suggested. The simplest perhaps is that strong selection for DDT resistance in the exposed heterozygous populations led to an increase in the frequency of genes favoring resistance. Somewhat different arrays of genes for resistance accumulated in 91 R and 731 R so that the F_1 hybrids carried a number of such genes whose combined effects conferred maximum resistance. In the unexposed controls no such accumulation had occurred, and the F_1 hybrids had intermediate resistance. Similarly, since fecundity was of relatively greater importance in the control populations, genes favoring increased fecundity were more strongly favored by selection, and in the F_1 hybrids their combined action led to the high fecundity observed. Though favored to some extent by selection in the exposed populations, fecundity was not of the same relative importance as in the controls so that the parental populations and their hybrids were less fecund than the comparable control populations due to the smaller accumulation of genes favorable to fecundity in the resistant populations. Similar reasoning may be applied to the fertility of the resistant flies, but while 91 C had a high fertility, the low fertility of 731 C, which appears to be real and not an artifact in the data, presents a puzzle for which there appears to be no simple answer.

The suggested explanation, of course, follows the lines of the dominance hypothesis described earlier, with the genes for greater resistance or fecundity at least partially dominant, and the heterosis being due to additive effects or to interactions among those genes. The absence of heterosis for resistance in the control F_1 hybrids is then attributable to the fact that genes for resistance have not increased in frequency due to selection in these populations.

SUMMARY

Flies drawn from two DDT resistant populations (731 R and 91 R) were crossed, and the original populations and their F_1 and F_2 hybrids were tested for DDT resistance, fecundity, and fertility. Similar crosses and tests were made

with the controls (731 C and 91 C). Heterosis was observed in the resistant F_1 hybrids for all three traits but only for fecundity in the F_1 control hybrids. F_2 values were in some cases significantly lower than the F_1, but there was no indication of "hybrid breakdown" in the F_2. Since each of the four originally heterozygous populations had been maintained in isolation for six years, evidence was sought for the development of coadapted genetic systems during this period, but no indications of coadaptation were found.

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LITERATURE CITED

EAST, E. M., 1936 Heterosis. Genetics **21**: 375–397.

FINNEY, D. J., 1952 A statistical treatment of the Sigmoid response curve. *Probit Analysis*. 2nd Ed. Cambridge University Press. Cambridge, England.

GOWEN, J. W., 1952 *Heterosis*. Iowa State College Press. Ames, Iowa.

HULL, F. H., 1945 Recurrent selection for specific combining ability in corn. J. Am. Soc. Agron. **37**: 134–145.

JONES, D. F., 1917 Dominance of linked factors as a means of accounting for heterosis. Genetics **2**: 466–479.

KEMPTHORNE, O. T., T. A. BANCROFT, J. W. GOWEN, and J. L. LUSH, 1954 *Statistics and Mathematics in Biology*. Iowa State College Press. Ames, Iowa.

MERRELL, D. J., and J. C. UNDERHILL, 1956 Selection for DDT resistance in inbred, laboratory, and wild stocks of *Drosophila melanogaster*. J. Econ. Entomol. **49**: 300–306.

REED, S. C., and E. W. REED, 1948 Natural selection in laboratory populations of Drosophila. Evolution **2**: 176–186.

SHULL, G. H., 1948 What is "heterosis"? Genetics **33**: 439–446.

WALLACE, B., 1958 The average effect of radiation-induced mutations on viability in *Drosophila melanogaster*. Evolution **12**: 532–556.

WALLACE, B., and M. VETUKHIV, 1955 Adaptive organization of the gene pools of Drosophila populations. Cold Spring Harbor Symposia Quant. Biol. **20**: 303–310.

THE RESIDUAL VARIABILITY IN INTENSITY OF COAT COLOR AT BIRTH IN A GUINEA PIG COLONY[1,2]

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A NUMBER of previous papers have been devoted to describing quantitatively the interaction effects of the major and certain minor color factors of the guinea pig (WRIGHT 1949, 1959b). We will consider here what can be said of the residual variability in intensity at birth in the guinea pig colony on which these studies have been based.

It will be convenient to repeat here the genotypes of the seven major series of uniform colors that have been recognized.

1. Dark-eyed dark sepia	*E aa B P* —
2. Pink-eyed pale sepia	*E aa B pp F*
3. Brown-eyed dark brown	*E aa bb P* —
4. Pink-eyed pale brown	*E aa bb pp F*
5. Pink-eyed pale brownish cream	*E aa* — *pp ff*
6. Stable yellow	*ee* — — — *F*
7. Fading yellow	*ee* — — — *ff*

Blanks in the succession of loci above imply that replacements make no appreciable difference in quality or intensity of coat color at birth. Eye color in the yellows (6,7) depends on the *B* and *P* loci (and others) in the same way as in the genotypes with *E*.

Each of these series varies continuously from a color of considerable intensity (especially in 1, 3 and 6) down to white. The principal genes concerned with these variations are in all cases those of the *c* series of alleles (C, c^k, c^d, c^r, c^a) in which C (dominant) determines the highest intensity with one important exception to be discussed here. With c^ac^a, *ee* c^rc^a, *ee* c^rc^r and *E* c^zc^z *ppff* (where c^z is any *c* allele except C) the coat is pure white at birth, irrespective of anything else (eyes pink with c^ac^a or *pp*, otherwise black (*B*) or brown (*bb*), slightly reduced in intensity with c^rc^r, much reduced with c^rc^a). Most other combinations of the *c* series give intermediate intensities, but in different orders in different cases. Heterozygotes among c^k, c^d, c^r, c^a are in general intermediate.

Replacement of *aa* by *A* (dominant) in the genotypes with *E* results in the presence of a subterminal phaeomelanic (yellow) band in otherwise eumelanic (sepia or brown) hair, the agouti pattern. The intensities of the two colors are the

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same as those due to *ee* and *E* respectively. The width of this band is subject to genetic variability but this has not been analyzed (except for an intermediate allele A^r, introduced into the guinea pig from the Brazilian cavy, *Cavia refescens* (DETLEFSEN 1914; WRIGHT in CASTLE and WRIGHT 1916).

Replacement of *E* by e^pe^p *or* e^pe results in the tortoise-shell pattern with a mixture of eumelanic and phaeomelanic areas in the coat, similar in color to those due to *E* and *ee* respectively. Gene e^p is incompletely dominant over *e* (CHASE 1939).

Another well-established pair of alleles is *S*, *s*. With *ss* and to a lesser extent *Ss*, there is a mosaic consisting of areas of color, usually of the quality and intensity determined by the rest of the genotype, and of pure white. There may also, however, be mosaicism in quality or intensity within the colored areas in certain cases (discussed in this paper).

There were second order variations in intensity at birth in the most intensively studied series, the dark-eyed sepias (1) and the stable yellows (6), that have been shown to be due largely to two additional loci—*Si,si; Dm,dm* (WRIGHT 1959a,b). The effects are cumulative with heterozygotes intermediate. The combination *sisidmdm* is pure white with reduced eye color even with *C* present, except for occasional small spots of pale color on the head. The combination *sisiDm–* tends to produce a nonprogressive sprinkling with white (silvering) especially with *C*. Gene *si* produces some dilution (in nonsilvered areas) in the presence of *C* but *dm* produces none. Both loci have similar but less thoroughly studied effects in the other series of colors. In the *pp* eumelanics (2,4,5) their effects are overshadowed by independent causes of variability, the analysis of which was not complete at the time at which the project had to be discontinued.

It is desirable to round out the analysis of the genetics of color in the colony in question as far as the data permit. The present paper is restricted to color intensity at birth and largely to the variation in the presence of *Si C*.

Grades of intensity

Three methods of grading have been described in previous papers. All animals have been assigned *visual grades* at birth, and often later, on the basis of standard squares of skin, chosen so that each grade is barely distinguishable from the adjacent ones (sepias: 1 to 21, browns: the same scale but rarely reaching 18, yellows: 1 to 13). Grade 0 is white (WRIGHT 1949).

Colorimetric determinations have been made of pigment from weighed hair samples from young animals of many genotypes (RUSSELL 1939; HEIDENTHAL 1940; WRIGHT and BRADDOCK 1949). These permitted quantitative scaling of the visual grades.

Finally galvanometer readings of intensity near the middle of the back have been obtained from animals of many genotypes by means of a reflectionmeter, using variously colored filters (amber, green, blue) (WOLFF 1955; WRIGHT 1959b). Separate indices were obtained for each filter by the formula $I_x = 10\,[\overline{log\ R_w} - log\ R_X]$, in which R_X is the reading on the live animal in question and

$\overline{log\ R_W}$ is the average logarithm of the readings of pure whites. These indices have been added to give a single index of intensity. This varies almost linearly with visual grade and can be transformed to give estimates of the latter and of relative amount of pigment (WRIGHT 1959b).

Analysis of variance

An analysis of variance will be made of the indices of the *SiC* genotypes of each major series of colors. This is of the following type in the simpler cases, letting I, M and T represent individuals, matings and total, respectively; n_I number of individuals, n_M number of matings, $\hat{n}_{I \cdot M}$ harmonic mean of numbers of individuals per mating etc. $\sigma^2_{I \cdot M}$ variance of individuals within matings etc.

	Degrees of freedom	Mean square	Variance component
I within M	$n_I - n_M$	$\bar{s}_{I \cdot M} = \sigma^2_{I \cdot M}$	$\sigma^2_{I \cdot M}$
M within T	$n_M - 1$	$\bar{s}_M = \sigma^2_{I \cdot M} + \hat{n}_{I \cdot M}\,\sigma^2_M$	σ^2_M
I within T	$n_I - 1$	$\bar{s}_I$	$\sigma^2_I = \sigma^2_{I \cdot M} + \sigma^2_M$

The variance within matings includes not only that due to genetic segregation but also any nongenetic variance within or between litters. The significance of differentiation among matings is tested in the usual way from the ratio of mean squares, $\bar{s}_M / \bar{s}_{I \cdot M}$. Significance would imply genetic differentiation if the numbers of individuals per litter and of litters per mating were uniform. Unfortunately, uniformity was not practicable. There were many litters in which only one individual of the genotype in question was graded, and many matings that were represented by only one litter. Matings represented by only one individual were, however, excluded.

It is necessary for interpretation to include litters (L) in the analysis. The following pattern is used.

	Dif.	Mean square	Variance component
I within L	$n_I - n_L$	$\bar{s}_{I \cdot L} = \sigma^2_{I \cdot L}$	$\sigma^2_{I \cdot L}$
L within M	$n_L - n_M$	$\bar{s}_{L \cdot M} = \sigma^2_{I \cdot L} + \hat{n}_{I \cdot L}\,\sigma^2_{L \cdot M}$	$\sigma^2_{L \cdot M}$
I within M	$n_I - n_M$	$\bar{s}_{I \cdot M}$	
M within T	$n_M - 1$	$\bar{s}_M = \sigma^2_{I \cdot L} + \hat{n}_{I \cdot L}\,\sigma^2_{L \cdot M} + \hat{n}_{I \cdot M}\,\sigma^2_M$	σ^2_M
I within T	$n_I - 1$	$\bar{s}_I$	σ^2_I

The differentiation of litters is tested by using the ratio $\bar{s}_{L \cdot M} / \bar{s}_{I \cdot L}$ and that of matings by using the ratio $\bar{s}_M / \bar{s}_{L \cdot M}$ except where $\bar{s}_{L \cdot M}$ is less than $\bar{s}_{I \cdot L}$ in which case $\bar{s}_M / \bar{s}_{I \cdot M}$ is used.

The nature of the population sampled is of great importance in interpreting the results. The color experiments did not consist of inbred lines (though brother-sister matings were often made) or even of closed strains. There were many variously designated strains within most of which several loci were allowed to segregate simultaneously, subject to the restriction that recombinations of interest be classifiable without question. The greatest cleavage of strains at the time at which the reflectionmeter readings were made was between a group in which segregation of *c* alleles (and only certain ones in any one strain) occurred in animals known to be all *FF*, and a group in which *f* (as well as *F*) was maintained in animals all with *C*.

Since animals were frequently introduced from one strain to another, within the above groups, all unselected modifiers must have been rather well distributed among them. In one heterogeneous strain, SH (genotype $(S,s)(E,e^p)$ *bb CC PP FF*), there was, however, selection of medium to strong dinginess of brown. This strain undoubtedly came to differ somewhat from most of the colony in modifiers of dinginess. In the many strains that carried *p*, the *pp* sepias and browns that were mated were, with rare exceptions, relatively intense after a period in which there was considerable confusion of classification from modifiers that could bring about overlapping of most of the *c* genotypes.

In a random breeding population, the genetic variance for a pair of alleles with intermediate heterozygotes is equally divided between that within and among matings. The arrays considered here, though drawn in most cases from several "strains", were much more like samples from a panmictic population than like an array from different inbred lines.

Some differentiation of litters was not unexpected because of the occurrence of differences in hair length and in condition of the fur that applied to all littermates. While individuals and often whole litters were not graded in cases in which poor condition was obvious, it is clear from the results that there were often small differences in condition among those that were graded.

In interpreting the variabilities that are considered here it should be recalled that it requires an index difference of about 2.3 units in most cases (1.5 in the case of black) for the difference in intensity to be barely recognizable to the eye. In no case was the standard deviation due to differentiation of litters as great as the minimum difference perceptible to the eye and in only two cases (pink-eyed pale browns, *FF* and *Ff*) was it more than half of this.

Residual variability of black

The blacks of genotype $Si(E,e^p)B\ C\ P$ show little variability to the eye (60 of grade 21, six of grade 20). This seems to be due to visual saturation at grade 21 since the relative variability of the amounts of pigment extracted per unit weight of hair (standard deviation of logarithms .067) is not much less than the average for the dark-eyed sepias of all of the various genotypes (.086) (WRIGHT and BRADDOCK 1949). The reflectionmeter readings of blacks are naturally very low and less reliable than for any other color. An analysis of variance was, however, carried through for the 66 indices of blacks of the above genotype after excluding

all with more than a trace of silvering and excluding matings from which only one offspring was graded. The harmonic means of numbers per litter and per mating were 1.393 and 2.678, respectively.

Black $Si(E,e^p)$ *B C P.* Mean 41.50

	DF	Mean square	F	Prob.	Variance components
I within *L*	27	.452	....	....	$\sigma^2_{I \cdot L} = .45$
L within *M*	18	1.501	3.32	<.01	$\sigma^2_{L \cdot M} = .75$
I within *M*	45	.871	....	....	
M within *T*	20	3.569	2.38	<.05	$\sigma^2_{M \cdot T} = .77$
I within *T*	65	1.701	....	....	σ^2_I 1.97

There is significant differentiation of litters within matings, and only somewhat doubtful differentiation of matings on allowing for differentiation among litters. All components and the total are small.

More than half (35) of those graded came from matings in which one or both parents carried *si*. There were ten graded young (mean 40.35) that were excluded because of having more than a trace of silvering and, thus, probably *sisi*. The mean grades of six tortoise-shells (e^pe^p) and 29 self-colored blacks (*E*) in the above group were 40.75 and 41.32, respectively. The means of 17 of genotype *EBCPFF* and 14 of genotype *EBCPff* from the other matings were 41.72 and 42.13, respectively. Thus, there are indications that *Sisi* (or even unrecognized *sisi*) and e^pe^p tended to reduce the index slightly. The *ff* blacks came from the same strain that produced the most intense browns and probably were affected by the same intensity modifiers.

Residual variability of nondingy dark brown

The browns of genotype $Si(E, e^p)bbCP$ present a more interesting situation than do the blacks. There is clear evidence for two kinds of genetic modifiers: (1) ones concerned with the upper limit of intensity, and (2) ones concerned with the peculiar sort of dilution known as dinginess. Silvering was not involved in the present data.

Table 1 excluding the bottom row shows the distribution of indices in a number of groups in which dinginess was absent, at least on the back. These include two groups of genotype *EbbCPpff* separated according to whether the mean index of the mating was above or below 35.00, one group consisting of those of genotype *EbbCPpF* and one consisting of the corresponding tortoise-shells, e^pe^pCPpF. Three groups with lower *c* combinations of approximately the same intensity as those with *C* are given for comparison.

The average index with *E* is higher than with e^pe^p, both in association with *CPpF* (difference .87 ± .53) and with c^kc^{kdr} (i.e., c^kc^k, c^kc^d, c^kc^r) (difference 1.47

TABLE 1

Distribution of reflectionmeter indices of nondingy intense brown, various genotypes and of dingy browns from strain SH

Genotype (all *bb*)	No. matings	Reflectionmeter index																		Index *I*		Transformed from *I*	
		22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	Total	Mean	SE	Visual grade	Pigment
Nondingy																								
ECPpff	7	..	..	..	..	..	..	..	..	..	..	1	..	..	3	6	10	1	1	22	36.78	.28	16.3	113.8
ECPpff	5	..	..	..	..	..	..	..	..	1	2	1	2	7	1	1	..	..	..	15	33.81	.41	15.0	93.7
ECPpFF	10	..	..	..	..	..	..	..	..	..	2	..	5	12	15	8	1	..	..	43	34.94	.19	15.5	100.9
e^pe^pCPpFF	6	..	..	..	..	..	..	..	..	..	2	1	3	1	3	2	..	..	..	12	34.07	.50	15.1	95.3
$Ec^kc^{kd\,r}PFF$	3	..	..	..	..	..	..	..	..	..	..	..	..	1	2	2	..	..	..	5	35.48	.39	15.7	104.6
$e^pe^pc^kc^{kd\,r}PFF$	8	..	..	..	..	..	..	..	..	..	..	3	3	3	3	..	..	..	.	12	34.01	.31	15.1	94.9
Ec^rc^rPFF	6	..	..	..	..	..	..	..	1	..	2	2	3	4	8	4	2	..	..	26	34.66	.37	15.3	99.1
E nondingy	31	..	..	..	..	..	..	..	1	1	6	4	10	24	29	21	13	1	1	111	35.11	.16	15.5	102.1
e^pe^p nondingy	14	..	..	..	..	..	..	..	..	..	2	4	6	4	6	2	..	..	..	24	34.04	.29	15.1	95.1
Dingy (SH)																								
ECCPPFF	8	2	3	2	..	3	3	5	3	2	3	2	1	..	..	..	..	..	..	29	27.88	.59	12.3	62.3

The mean indices and the corresponding visual grades and relative amounts of pigment of 198 nondingy browns (*Ee^{p}bbCP*) with mean index 34.8 are taken as 100 as in a previous paper (WRIGHT 1959b).

± .50). The grand average for nondingy browns with *E* exceeds that for those with $e^p e^p$ by 1.07 ± .33.

An analysis of variance was made of the 76 indices in the first three rows (*EbbCPp*) after excluding matings from each of which only one animal was graded. The harmonic means for individuals per litter and per mating were 1.774 and 3.130, respectively.

Nondingy brown *SiEbbCPp*. Mean 35.24

	DF	Mean square	F	Prob.	Variance components
I within *L*	42	1.038			$\sigma^2_{I \cdot L} = 1.04$
L within *M*	16	2.423	2.33	<.05	$\sigma^2_{L \cdot M} = .78$
I within *M*	58	1.420			
M within *T*	17	8.409	3.47	<.01	$\sigma^2_M = 1.91$
I within *T*	75	3.004			$\sigma^2_I = 3.73$

The estimated variance of litters within matings is small and about the same as in blacks but is less certainly significant because of the more than doubled variance of individuals within litters. The estimated variance among matings is highly significant and nearly 2.5 times that estimated for blacks.

Intense brown has only about half as much eumelanin as intense black. There is a general parallelism in the effects of the lower *c* combinations on the two colors but relatively (as well as absolutely) much less spread among the browns. Thus, $c^r c^a$ and $c^d c^a$ browns (with *SiSiDmDm*) have about 75 percent as much pigment as nondingy *C* browns in contrast with 46 percent in the corresponding comparison in sepias. Similarly, $c^r c^r$, $c^k c^{kdr}$ and nondingy *C* browns are indistinguishable in coat color, while $c^r c^r$ dark sepias are obviously lighter than *C* blacks, and $c^k c^{kdr}$ can usually be distinguished from the latter on comparison of live animals. These comparisons indicate, as noted in previous papers, that the reduction to about 50 percent of the quantity of eumelanin from black to brown is associated with imposition of a more drastic limit on the amount of pigment that can be produced by excess tyrosinase (the effectiveness of which is determined primarily by the *C* alleles) (Russell 1939; Ginsburg 1944; Foster 1956). Thus, there is more damping of the effects of the lower *c* alleles by approach to a ceiling in the browns than in the sepias. The replacement of *P* by *pp* reduces the amount of eumelanin of blacks much more than does replacement of *B* by *bb*, but there is no indication of imposition of a limiting factor on the effects of the lower *c* alleles. These, on the contrary, show a much greater relative spread than in dark sepias. The replacement of *B* of *BppF* sepias by *bb* changes the quality to pale brown but reduces the amount of pigment only slightly. The relative spread among the effects of the lower *c* alleles is almost as great as in the pale sepias. It has been concluded that with *ppF* there is greatly reduced efficiency of the tyrosinase due to a given *c* combination instead of imposition of a lower ceiling on its activity as with *bb*.

The data of Table 1, and the analysis of variance, indicate that the height of the ceiling varies considerably, and that this is to a large extent under genetic control. The seven matings in the first row of Table 1 produced only one offspring in 22 with index below 35.00, while the five matings of the second row (of the same genotype in all major factors) produced only two offspring in 15 with index above 35.00. The former group produced on the average 21 percent more pigment than the latter. There is, however, no clear evidence of segregation of a major modifier within matings.

The most plausible interpretation of this aspect of the residual genetic variability of browns is that there are multiple loci that affect the level of the physiological limiting factor for intensity. This can have nothing to do with the limiting factor responsible for dominance of *C* since this is found whether brown (or any other color) is near its ceiling or not (e.g., in pale brown *EbbCppF*). Dominance must be due to a physiological limit that applies directly to the *C* product.

It is probable that $e^p e^p$ sometimes lowers the ceiling for brown. It will be convenient to use the symbols Lb_1, Lb_2, etc., and collectively $\Sigma(Lb)$ (limited brown) for the plus phases of the other loci (hypothetical) that affect intensity within *EbbCP*.

The variations in intensity among browns with $c^k c^{kd^r}$ and $c^r c^r$ are presumably due to these same modifiers. Lesser damping effects would be expected with the still lower *c* combinations, but in these the effects are confounded with the increasing effects of *Si,si* and *Dm,dm* on intensity.

It is probable that the same modifiers, $\Sigma(Lb)$ also affect the limiting intensity of black.

Analysis of the variability of dingy browns

The second type of variation in browns is wholly restricted to those that carry *C*. With a low grade of dinginess (WRIGHT 1947) there are scattered hairs, restricted to the cheeks and nape, that are as light as pink-eyed pale browns either in a subterminal band or throughout their lengths, except for two or three millimeters of intense brown at the tip. In higher grades the proportion of such hairs is greater in the regions referred to and there is a sprinkling of them on the back, especially anteriorly. In the highest grades all hairs are affected over most of the coat. The rump, nose and feet are least affected.

Animals of the highest intensity in unaffected hairs may show scattered hairs of the above type. The term "dinginess" is not an appropriate one for these attractively silvered intense browns. High ceiling intensity seems, however, to be incompatible with the higher grades of dinginess. The term is most appropriate where there is a mixture of dark-tipped pale-brown and uniform dull-brown hairs. No white could be found in the affected hairs of even the palest dingy browns in this colony.

As described in a previous paper (WRIGHT 1947), a strain (S) was established in which all animals were dingy browns, most of them of high grade, by selection of such animals as parents. This strain was continued as SH into the period in which reflectionmeter indices were obtained. There was occasional introduc-

tion of strongly dingy animals from other strains. This strain came to be of constitution (S,s) (E,e^p) *bbCCPPFF*.

The reflectionmeter index from the darkest region of the back is a very imperfect measure of this character since animals with slight to medium dinginess may show no reduction of the index. Nevertheless, as shown in Table 1, bottom row, the distribution of indices for strain SH contrasts markedly with distribution for nondingy intense browns. The mean index of the former 27.88 ± .59 contrasts with 35.23 ± .19 for *EbbCPp* in Table 1. This implies only about 60 percent as much pigment. The amount of pigment extracted from weighed samples of strongly dingy hairs was 42 percent of that in samples of nondingy intense brown hairs. (Analyses by DR. A. S. FOX).

The analysis of variance is given below for the 74 animals of this genotype that came from matings from which at least two young were graded. The harmonic mean of individuals per mating was 3.884.

Dingy brown, *EbbCCPPFF*. Mean 25.63

	DF	Mean square	F	Prob.	Variance components
I within *L*	47	16.000			
L within *M*	11	9.275	1.72	>.05	
I within *M*	58	14.725			$\sigma^2_{I \cdot M} = 14.73$
M within *T*	15	49.849	3.38	<.001	$\sigma^2_M = 9.04$
I within *T*	73	21.942			$\sigma^2_I = 23.77$

In this case the differentiation among litters within matings is less than expected by chance though not significantly. The differentiation of matings, on the other hand, is significant at the .001 level. The two components of variance are enormously larger than in the nondingy browns or blacks. There is undoubtedly a considerable nongenetic component in the variance of individuals, but the large variance among matings makes it certain that genetic modifiers played a large role in this strain.

An attempt was made to produce a weakly dingy or nondingy strain (SL) of the same major genotype (*EbbCCPPFF*) at the time of establishment of strain SH, but all such animals that were tested turned out to be *Pp*, already known to reduce dinginess greatly (WRIGHT 1947). Later, outcrosses of SH were made to animals from various sources believed to be *PP*. In two cases a few nondingy browns of genotype *EbbCPPFF* were obtained.

In one of these, a male with very strong dinginess from strain SH (mating SH83) was mated with an unrelated black of genotype *EBbCPPFF*. All of the five offspring that were brown showed much less dinginess than any of the nine offspring of SH83. They showed slight to medium dinginess on the head but no more than a trace on the back, too little to affect the index (average of three, 34.0). A brother-sister mating, SN39, produced an F_2 progeny of 19 individuals.

None of these showed more than the weak dinginess of F_1. Four showed mere traces on the head, and two showed none at all. The average index for 13 was 34.45 ± .49. One of the two with no dinginess was mated with a pink-eyed pale-brown male (*EbbCppFF*). The 18 offspring were all nondingy dark browns (*Pp*) except for one with a trace of dinginess on the head. The mother was the first clear case of nondingy *EbbCPPFF* to be observed. Two F_3 matings were made. One from very slightly dingy parents produced three offspring like themselves. The other came from parents that were the dingiest of the weak dingies of F_2. One of the four offspring was only slightly dingy, but the other three were as strongly dingy as the average of SH83. Thus, while strong dinginess did not appear in F_1 or F_2, it did in F_3. These results show that genotype *EbbCPPFF* can vary from the highest degree of dinginess to none at all, but that the difference between extremes, or even that between nondingy and dingy collectively, must have been multifactorial.

In the other outcross (SN41) a female with relatively slight dinginess for strain SH (from a brother-sister mating from SH83) was mated with an unrelated dilute brown ($e^p ebbc^d c^a PPFF$). Eight of the 35 offspring in this case showed dinginess fully typical of strain SH, 22 showed no more than in the preceding F_1 and F_2, three showed mere traces on the head and two were nondingy. The average index of 16 (including two strong dingies) showed little depression (34.35), but it is clear that this outcross was to an animal that was heterozygous in genes favoring dinginess. Two F_2 matings were made, both from parents that showed only weak dinginess restricted to the head. In both cases, the offspring covered the range from very strong dinginess to none at all. Unfortunately the nondingies were of doubtful genotype since one of the matings produced $c^d c^a$ and the other $c^d c^d$ segregants, neither of which ever show a trace of the character. These are not as intense as browns with *C* but there is overlap at least in the case of $c^d c^d$. The most intense of the F_2 nondingy young (index 34.8) was tested by mating with a pink-eyed pale sepia of genotype $EBbc^d c^a ppFF$. All of the 27 young were dark-eyed (*Pp*) and seven were blacks (*C*) proving that this tested nondingy F_2 female was *EbbCPPFF*, and thus, the second case in which it was proved (by breeding tests) that this genotype may be nondingy.

In this second outcross, there appeared to be segregation of one or more, probably two, major modifiers. There is agreement with the first outcross, however, in the indication that no single locus controls the difference between nondingy and dingy, collectively, in browns of genotype *EbbCPPFF*. We may postulate an array of modifiers Db_1, Db_2, etc., collectively $\Sigma(Db)$, that vary considerably in effect.

Mosaicism of dingy brown due to gene s

A reading was taken on the lightest as well as on the darkest region of the back in most of the animals of strain SH. Table 2 shows the means of the maximum index in all animals in which a reading was taken and both the maximum and minimum for all on which both were taken. A subdivision is made according to the absence (*E*) or presence (e^p) of yellow spotting and according to the amount

TABLE 2

Mean maximum and minimum indices of classes of dingy browns of strain SH classified by absence (E) *or presence* (e^pe^p) *of yellow spotting and by amount of white (none, white present but not on back, and white on the back). Mean maximum indices are given for all graded and for those with a minimum index*

E, e^pe^p	Amount of white	No.	Maximum grade: All graded $\bar{I}$	Maximum grade: All graded SE	Maximum grade: With min. grade $\bar{I}$	Maximum grade: With min. grade SE	Minimum grade $\bar{I}$	Minimum grade SE
E	no W	29(25)	27.9	0.6	(27.6	0.7)	19.9	0.8
	little W	8(6)	24.0	2.0	(22.5	2.4)	15.9	1.8
	W on back	23(18)	25.1	1.2	(26.2	1.0)	18.2	1.6
	Total E	60(49)	26.3	0.6	(26.5	0.6)	18.8	0.8
e^pe^p	no W	2(2)	26.9	..	(26.9	..)	18.5	..
	little W	3(2)	22.9	..	(22.5	..)	16.7	..
	W on back	14(8)	22.7	0.9	(22.4	1.2)	16.1	1.1
	Total e^pe^p	19(12)	23.2	0.8	(23.1	1.0)	16.6	0.9

TABLE 3

Mean maximum and minimum of index, visual grade (VG) and amount of pigment (Pig.) (VG and Pig. by transformation of I) for the dingy browns of strain SH, classified by E, e^pe^p *and by presence or absence of any white*

E vs. e^pe^p	Amount of white	Maximum grade: No.	I	VG from I	Pig. from I	Minimum grade: No.	I	VG from I	Pig. from I	Percent pig. min. / max.
E	no W	29	27.9	12.3	62.3	25	19.9	8.8	33.7	54.0
	W present	31	24.8	11.0	49.7	24	17.6	7.8	27.7	55.7
e^pe^p	no W	2	26.9	11.9	58.0	2	18.5	8.2	29.9	51.5
	W present	17	22.7	10.1	42.4	10	16.2	7.2	24.3	57.3

of white: "no W" if no white, "little W" if some white but none on the back and "W on back" if white on the back. There is no indication of bias from the failure to obtain a minimum index in all cases.

No clear indication of any relation of dinginess to white or to yellow spotting was found at the time of the 1947 paper on the basis of the averages from arbitrary grades of the character. The data in Table 2 suggest, however, that both tend to reduce intensity somewhat, though not wholly conclusively, because of heterogeneity and because of a possible effect of the restriction of the sites at which readings could be taken in the case of the tortoise-shells.

This probable effect of e^pe^p may be interpreted as a by-product of an occasional lowering of the ceiling for brown, which as noted favors dinginess.

There is no consistent difference between those with white on the back and those with white restricted to other parts of the coat. These two categories are combined in Table 3 which shows not only the mean indices for darkest and lightest areas on the back but also the mean visual grades and mean relative

amounts of pigment both estimated by transformation of the indices. The amount of pigment estimated for 198 nondingy browns, *EbbCPFF*, mean index 34.8, is taken as 100 as in the previous paper (WRIGHT 1959b).

It is interesting that the ratio of amount in the lightest area to that in the darkest is about the same (average 55 percent) in all four categories. This contrasts with the approximate uniformity of the intensity of brown on the back of nondingy browns. There was a correlation of .81 ± .07 between maximum and minimum in the self-colored browns and of .75 ± .09 in those with white in the coat. It may be noted that the amount of brown pigment is reduced to one third of that of intense brown in the lightest region in self-colored dingies and to about one fourth in tricolors.

While the effect of *s* on the mean index is slight at best, there is clear evidence from mere inspection of spotted dingies that there is an effect of another sort. In self-colored dingies of strain SH, there was almost always an orderly symmetrical pattern. As already noted there was usually strong dinginess on cheeks and nape but little or none on the nose. In about 25 percent a transverse line back of the shoulders sharply separated a light anterior region from a darker posterior one. In most of the others, there was a gradual transition from light to dark in this region, but in a few there was little change. In about a third, gradual darkening continued posteriorly culminating in brown of almost full intensity at the extreme posterior end where readings were never taken. The belly showed considerable dinginess as a rule but not the legs.

Among 43 self browns of this strain in which the pattern was carefully described in a rubber stamped outline, there was appreciable asymmetry in only one (strong dinginess in an area on the left side of the back opposite moderate dinginess on the right side).

The contrast between these self browns and those with any white in the coat is brought out in Table 4 (which is restricted to browns with no yellow spotting). About 40 percent of the 81 with a little white but none on the back and 48 percent of the 102 with some white on the back showed marked bilateral asymmetry with respect to degree of dinginess.

A second relation to white is also conspicuous. In 27 percent of those with some white but none on the back, and in 53 percent of those with white on the back,

TABLE 4

Pattern of dingy browns of strain SH classified according to amount of white as in Table 2 and according to whether differences in grade of dinginess show a relation to white, if present, and according to bilateral symmetry or asymmetry

	Amount of white	No relation to white		Relation to white		Total	Percent asym.	Percent relation to white
		Sym.	Asym.	Sym.	Asym.			
E	none	42	1	..	..	43	2.3	...
	little W	47	12	2	20	81	39.5	27.2
	W on back	43	5	10	44	102	48.0	52.9
	Total *E*	132	18	12	64	226	36.3	33.6

there were abrupt intensity boundaries that were clearly related to the pattern of color and white. The commonest relation was a pronounced difference in grade of dinginess on opposite sides of the head, separated wholly or in part by a narrow white nose streak. In other cases two adjoining areas of markedly different degrees of dinginess on the back were separated in part by a sharp boundary which ran into the point of a wedge of white which continued the separation of grades of dinginess. In some cases such a boundary, continued as a white streak, might lie transversely across the back so as to give a symmetrical pattern, but more frequently it lay diagonally or longitudinally, giving asymmetry. In Table 4, each animal is entered only once although some showed such peculiarities, as described, in more than one part of the coat. Thus, gene *s* clearly brings about a tendency toward mosaicism within the colored areas, and it is clear that this is related developmentally to the mosaic pattern of color and white that is its most obvious effect. In spotted nondingy browns there is, on the other hand, no indication that *s* brings about any tendency toward mosaicism of intensity.

This modification of the dingy pattern by *s* is much like that which *s* causes in the tortoise-shell pattern (WRIGHT 1917, 1923; ILJIN 1928; CHASE 1939). In tortoise-shells that have no white (SSe^pe^p) there is usually a mere sprinkling of yellow hairs on a eumelanic ground. In tricolors (sse^pe^p), the pattern of irregular blocks of color on a white ground is the same as in piebald bicolors (*ssE, ssee*), but, instead of these blocks being all tortoise-shell, some tend to be largely or wholly eumelanic and others largely or wholly phaeomelanic. Such blocks may be wholly separated by a white streak or by a sharp boundary that runs into a white streak or at least into the point of a wedge of white.

While eumelanin and phaemelanin are distributed in the main as equal alternatives in the tricolor pattern, there are many cases in which eumelanic head spots on a phaeomelanic ground simulate the typical relations of color to white on the head in piebalds. There are also moderately frequent cases in which a narrow streak between two eumelanic blocks is white at one end, yellow at the other. There are few, if any, cases of the reverse relation between the two colors. Thus, eumelanin is related to phaeomelanin within the colored areas somewhat as color, collectively, is related to white.

This raises the question whether there is a similar relation between grades of dinginess. An examination of the recorded patterns of strain SH revealed nine cases in which a narrow streak of dark brown separated, at least in part, two large blocks of light (strongly dingy) brown. In two of these the dark brown streak was a continuation of a white streak, and in two others it was a continuation of a yellow streak. There were also two cases (not included above) in which a double streak (dark brown along one side, white on the other) separated light brown areas. There were eight cases in which a large light-brown area had a narrow dark-brown border next to a large yellow area. In only one doubtful case was a reverse situation to any of these found: white continued by light brown between dark-brown areas.

There were many cases in which light-brown cheek spots were set off sharply from a dark ground, but this differs from the situation in unspotted slightly dingy

animals only in the sharpness of the boundaries. Related to this was a large number of cases in which a white nose streak was continued on the nape by a small area of dark brown (instead of the more usual white spot) both surrounded by light brown. It seems clear that in mosaics due to *s*, the *light*-brown areas are related to the *dark* ones in the way that eumelanin, collectively, is to yellow or as color, collectively, is to white.

It may be added that mosaicism due to *s* is much less frequently manifested in other colors. It is occasionally responsible for blocks of sharply different intensity of pale sepia or pale brown, separated wholly or in part by white streaks. As noted in the previous paper, it seems to be responsible in some cases for blocks of different grades of silvering in animals with *sisi*. Still more rare are cases of apparent somatic mutation of major color factors in which the "mutant" spots are related to white in a similar way to those above. Such a relation has been noted in other mammals (cf. CASTLE 1929; DUNN 1934 on such cases in rabbits and mice respectively). It should be said that a few apparent somatic mutations have been observed in which *s* was absent.

Relation of genes C, P *and* F *to dinginess of brown*

Earlier studies of dinginess (WRIGHT 1947) were based on outcrosses of the dingy strain S (genotype (S,s) (E,e^p) *bbCCPPFF*). A systematic attempt to introduce the character into brown-dilute yellow tortoise-shells carrying c^kc^k instead of *C* was made by repeated backcrossing to tortoise-shells of strain S followed by inbreeding in order to extract ones with dilute yellow spotting. The resulting strain (SK) was maintained by crosses of $Cc^k \times c^kc^k$ and $Cc^k \times Cc^k$. The brown-*red* segregants (*C*—) showed moderate to strong dinginess as in strain S while no trace of dinginess was ever observed in the brown-*yellow* segregants (c^kc^k). Less systematic observations of lower *c*-combinations indicated that *C* is necessary for any dinginess whatever. This conclusion has been fully substantiated by all later observations.

An attempt to introduce dinginess into pale browns (*bbCCppFF*) also completely failed. These experiments (strain SP) showed moreover that PP is necessary for more than a trace of dinginess on the back or for strong dinginess on the head. Matings of $PP \times pp$ or of $Pp \times pp$ have never produced young with strong dinginess in spite of high concentration of dingy ancestry of strains S or SH, while matings between *Pp* offspring of such matings produced strong dinginess in the proportion expected of *PP*. Numerous breeding tests of strong and weak dingies from $Pp \times Pp$ have shown the former to be *PP* and the latter *Pp*.

An attempt to introduce dinginess of strain S into brown-dilute yellow tricolors of genotype $sse^pe^pbbCCPPff$ was more successful, but among 203 of this genotype with high concentration of S ancestry, only three showed dinginess of the highest grade. In a strain (SF) maintained by matings $Ff \times ff$ and $Ff \times Ff$ (as well as $ff \times ff$), the brown-*red* segregants (*F*—) showed significantly stronger dinginess than the brown-*yellow* ones (*ff*). This strain continued into the period in which reflectionmeter indices were obtained. The average for 13 brown-reds (all *Ff*) was 26.9 ± 1.5 and for 31 brown-yellows (*ff*) was 30.9 ± 0.6. The difference is

significant. No clear evidence has been obtained, however, of a difference between *FF* and *Ff*.

The combination *ECPpff* has been produced in considerable numbers (Table 1). These have shown no dinginess at all except for mere traces on the head in two cases.

No clear evidence has been found of a difference at birth between *Cc^{z}PPFF*, which has frequently been of the highest grade of dinginess, and *CCPPFF*. It was found, however, that the former darken significantly more with age (WRIGHT 1947). There is some indication that *Cc^{z}PPFf* tends to have less dinginess than comparable *CCPPFF*, but one clear case of strong dinginess (index 26.7) has been obtained. Genotype *Cc^{z}PpFF*, on the other hand, has never shown more than a trace of dinginess and, thus, less than *CCPpFF*.

It is clear that the system of major genes that control the intensity of brown pigmentation (*Ebb* present) is one that has an optimum. The combination *c^{k}c^k-ppff* is pure white. *Cppff* has a trace of brown; *c^{k}c^{k}ppFF* and *CppF* are pale browns with the latter more intense, and *c^{k}c^{k}P—* is of full intensity. The observed maximum is with *CPpff*. With a given array of modifiers, Σ(*Db*), *CPpFf*, *CPPff* and *CCPPFF* are progressively lighter, the last becoming as pale as *CppF* except at the tip of the hair, with sufficient minus modifiers.

IBSEN (1932) and IBSEN and GOERTZEN (1951) have described a color variation (called "white tipped" in the former reference; "whitish" in the latter reference) that is clearly related physiologically to dinginess. In whitish browns, a few to most of the hairs are affected. The tip is always intense (according to the later paper), but below the tip the hair may be pure white, or there may be one or more bands of pure white separated by dilute brown. Other hairs are merely of the dingy type, dilute with dark tip. The data clearly indicated the presence of a gene *W* which causes many of the hairs of intense browns (*EbbC*) to be whitish in homozygotes (*WW*) but only a few in heterozygotes (*Ww*) and none with *ww*, which, however, usually is dingy brown. Like dinginess, the character was shown to require the presence of *C*. No mention was made of a relation to *P*,*p* or *F*,*f*, but *f* was probably wholly absent from the colony, and as no mention was made of segregation of *p*, this was probably absent in the experiments on whitish. The whitish character disappeared with age in some two thirds of the females and one third of the males which agrees with the behavior of dinginess to the extent that females darken with age more than do males (WRIGHT 1947). It differed from dinginess in that it could be transferred to blacks, but only in homozygotes (*WW*) and in these only in a few hairs on the head which were soon replaced by wholly black hairs.

It appears that *W* is a modifier of dinginess that was wholly absent from the colony discussed here, since even the strongest dingies never showed any white in the lightest brown hairs.

The peculiar genetics of brown may be interpreted in various ways. The following scheme was suggested in a previous paper (WRIGHT 1949). "With regard to dinginess of brown, the simplest interpretation—is that the product of the *C*, *P* and *F* series combine with some other factor to produce the eumelanic enzyme

system and that this reaction is practically quantitative up to the limit determined by the amount of this factor. It is supposed that this limit is reached in genotype Cc^aPpff and that the excess of *CPF* product in higher genotypes gives rise to an inhibitor which tends to transform the brown (but not the sepia) substrate in a way that does not lead to pigment. It is further supposed that this diversion takes precedence almost quantitatively over the competing process of transformation to brown pigment".

In the light of IBSEN and GOERTZEN's evidence that the gene *W* when homozygous and associated with *CCPPFF* may produce a slight inhibition even in black, it may be postulated that in addition to a qualitative difference gene *B* doubles the amount of the limiting factor found with *bb* and so prevents excess *CPF* product and production of inhibition in blacks except under the most extreme conditions.

With respect to the competition postulated between inhibitor and the brown enzyme system, it cannot be maintained that pigment substrate (tyrosine) is a sharply limiting factor since its rate of production is great enough to maintain the formation of black pigment in twice the amount of the most intense brown. Under the above view it must be supposed that the inhibitor (present only with excess *CPF* product) is so much more effective in competition with the brown enzyme system that the amount of tyrosine available for formation of brown pigment may be reduced far below half of that in the absence of the inhibitor. This is a rather extreme requirement.

There is no necessity that the second competitive phase be in the reaction with tyrosine. It may occur within the formation of the enzyme system itself. This possibility is represented diagrammatically in Figure 3. It is supposed that the *CPF* products interact with the limiting factor (high in amount with *B*, only half as high with *bb*) to produce the corresponding enzyme system. If, however, the *CPF* products are still in excess as the limiting factor approaches exhaustion, they proceed to modify the enzyme system in such a way that it is no longer effective *in vivo*, with dinginess as the consequence.

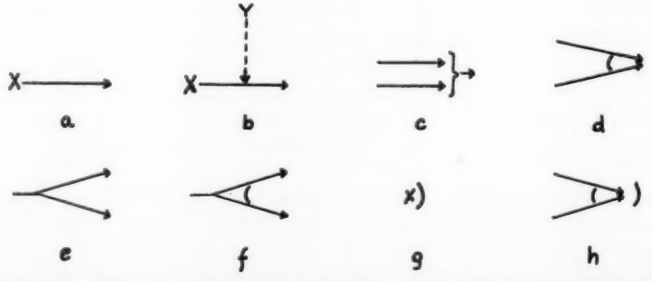

FIGURE 1.—Key to Figures 2 and 3: (a) process controlled by gene *X*; (b) process (due to *X*) modified by another (due to *Y*); (c) additive processes; (d) a joint reaction; (e) nonalternative effects; (f) alternative effects; (g) an inactive gene; (h) an inactive reaction product. The relative extents of reactions are indicated in Figures 2 and 3 by the heaviness of the lines and by numerals.

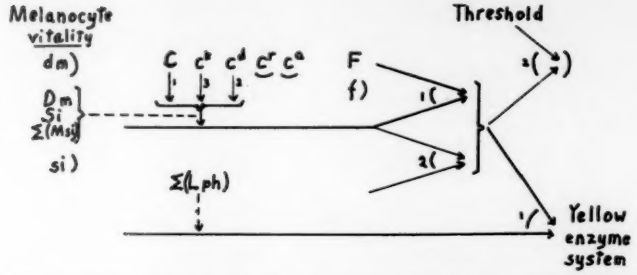

FIGURE 2.—Hypothetical processes leading to the formation of the yellow producing enzyme system in melanocytes with phaeomelanic differentiation. These include all in hair follicles (at birth) in genotype *ee*, many in e^pe, relatively few in e^pe^p, subject to modifiers, especially *S,s*. Also included are the melanocytes in an intermediate phase of the follicle cycle in EA etc. Presence or absence of *P,p* Σ(*Mp*) or *B,b* are without effect. The occurrence of thresholds is shown especially by the absence of dorsal pigment in $eec^{kd}c^{ra}ff$, presence in $eec^{kd}c^{kd}ff$. The limiting capacity to form yellow is enormously higher in guinea pigs than in wild *Cavia cutleri*, because of unanalyzed modifiers Σ(*Lph*).

The qualification "*in vivo*" seems to be required by studies of the dopaoxidase activity of colorless extracts from skins of new born guinea pigs of various genotypes by GINSBURG (1944) and the tyrosinase and dopaoxidase activity of homogenates of fetal guinea pig skin of various genotypes by FOSTER (1956). GINSBURG found somewhat higher activity in forming melanin in extracts from browns (*EbbCPPFF*) than in extracts from any other genotype including black. Nearly all of his browns were strong dingies of strain S. Similarly FOSTER found higher oxygen consumption and melanin formation from both tyrosine and dopa by homogenates from browns than from blacks or any other genotype. Half of his browns were strong dingies from strain SH (*EbbCCPPFF*), others were very intense browns of genotype *EbbCCPpff*. There was no consistent difference. Thus, whatever the basis for the inhibition manifested in dingy browns, it does not affect tyrosinase or dopaoxidase activity *in vitro*.

The residual variability of stable yellow

Following is the analysis of variance of the indices from intense yellows ("reds") of genotype *Si*(*e*,e^p)*CFF*. All matings from which *sisi* might segregate are excluded. The harmonic means of individuals per litter and of individuals per mating were 1.486 and 3.240 respectively.

There is very little variability within litters and no significant differentiation of matings on taking account of the small but highly significant differentiation of litters within those matings that were represented by more than one litter. There is clearly no residual genetic variability of importance.

This does not mean that such variability may not occur in other colonies. In fact at a much earlier period, the present colony included an inbred strain (No. 32) of genotype $SiSie^pe^pCCFF$ that was of so intense a brownish red that visual

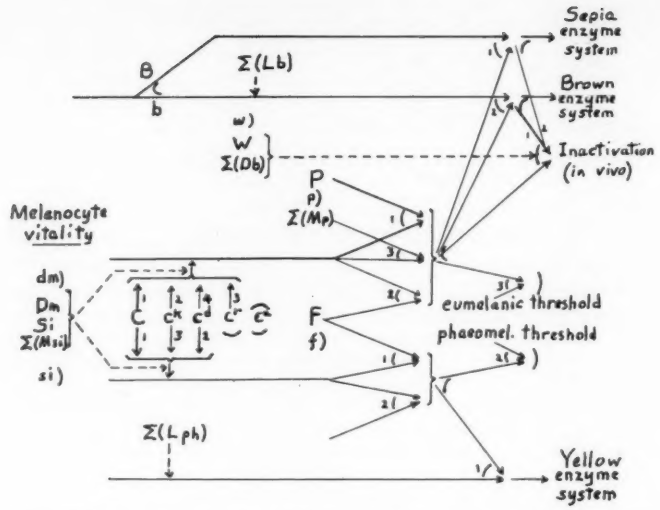

FIGURE 3.—Hypothetical processes leading to the formation of pigment producing enzyme systems in melanocytes with eumelanic differentiation. These include all in hair follicles in genotype *Eaa*, most in $e^p e^p aa$, relatively few in $e^p eaa$, subject to the effects of modifiers, especially *S,s*. Also included are the melanocytes in early and late phases but not an intermediate phase of the follicle cycle in EA, etc. All melanocytes in skin and eyes (in which $c^d > c^r$) are included. A feeble underlying potentiality for producing yellow becomes manifested in follicles in genotype *ECppff* and in genotypes with c^d (especially $Ec^d c^d ppF$). The occurrence of both eumelanic and phaeomelanic thresholds is shown by the complete absence of any pigment in $Ec^k c^k ppff$ in comparison with the presence of both sorts in *ECppff*, and in other cases. Inactivation of the brown enzyme system occurs only in $E(e^p)CP$ and is greatest in $E(e^p)CCPPFF$ with favorable modifiers $\Sigma(Db)$ and *W*. Inactivation of the sepia enzyme system occurs only under the most extreme conditions (including presence of *WW* (which was absent from the colony discussed here, but present in IBSEN's colony).

Intense yellow, $Si(e, e^p)CFF$. Mean 22.90

	DF	Mean square	F	Prob.	Variance components
I within *L*	21	.410			$\sigma^2_{I \cdot L} = .41$
L within *M*	17	2.241	5.47	<.001	$\sigma^2_{L \cdot M} = 1.23$
I within *M*	38	1.229			
M within *T*	9	3.062	1.34	>.05	$\sigma^2_M = .25$
I within *T*	47	1.580			$\sigma^2_I = 1.89$

grade 13 was regularly assigned to individuals in contrast with grade 10 in other strains of the same genotype in known major genes (e.g., inbred strain No. 2). This implies about twice as much phaeomelanin in strain 32 as in strain 2. Crosses between these strains produced young of intermediate intensity. There

was no recognizable segregation in F_2 which indicates that the difference was due to multiple minor factors.

Wild Peruvian cavies (*Cavia cutleri*), which produce fertile hybrids with the guinea pig and are believed to be ancestral, have a cream color in their agouti pattern which can hardly have more than one fourth as much phaeomelanin as in a typical golden agouti guinea pig (*CCFF*). Yet they have the same genotype with respect to major known genes. They have been shown to possess *C* rather than a lower allele by crosses with albino guinea pigs ($c^a c^a$). Gene *f* was not known at the time but the absence of unifactorial segregation indicates *F*, (CASTLE in CASTLE and WRIGHT 1916). It is possible that they were *dmdm* (WRIGHT 1959b), but *Dm,dm* have no visible effect in intense yellow guinea pigs. Thus, there must be multiple modifying factors $\Sigma(L\ ph)$ that can cause at least an eightfold difference in the amount of yellow pigment within genotype *SiSieeCCFF* but these were absent in the strains analyzed here.

A separate analysis of variance was made of the light reds with *Ff* instead of *FF*. The harmonic means of individuals per litter and per mating were 1.635 and 2.797, respectively.

Light red, *SiSieeCCFf*. Mean 20.71

	DF	Mean square	F	P	Variance components	
I within *L*	20	1.119			$\sigma^2_{I \cdot L} = 1.12$	
L within *M*	6	1.628	1.46	>.05	$\sigma^2_{L \cdot M} = .31$	
I within *M*	26	1.237				$\sigma^2_{I \cdot M} = 1.24$
M within *T*	9	7.331	4.50	<.05	$\sigma^2_M = 2.04$	$\sigma^2_M = 2.18$
I within *T*	35	2.804			$\sigma^2_I = 3.47$	$\sigma^2_I = 3.42$

The situation is very different from that with the *FF* reds. There was clearly appreciable residual genetic variability. It is possible that the same genes were present but ineffective with *FF* in the strains from which the *FF* reds were drawn, but this is not certain because of the separation of the strains for several years. The data do not permit any analysis in terms of specific genes.

The variability of yellows with various combinations of lower *c* alleles has been analyzed in previous papers (WRIGHT 1959a,b) as due largely to *Si,si; Dm,dm* of which the former pair also affects the intensity of *C* yellows while the latter pair does not. The identification of the genotypes of individuals in these terms is too uncertain to permit analysis of residual variability which in any case was slight.

It may be noted here, however, that there was one small cluster of matings in which there was segregation of abnormally high intensity of $eec^d c^a FF$ (visual grade 6 as opposed to grade 4 or 5 of littermates) for several generations after an outcross to another color. This shows that additional modifiers exist.

The residual variability of fading yellow

The analysis of variance of yellows of genotype *SiSieeCCff* is based on animals from the same strains as those from which *Ff* light reds were taken. The harmonic means of individuals per litter and per mating were 1.300 and 2.509, respectively.

Fading yellow, *SiSieeCCff*. Mean 13.51

	DF	Mean square	F	P	Variance components	
I within *L*	16	1.008			$\sigma^2_{I \cdot L} = 1.01$	
L within *M*	13	1.078	1.07	>.05	$\sigma^2_{L \cdot M} = .05$	
I within *M*	29	1.039				$\sigma^2_{I \cdot M} = 1.04$
M within *T*	12	5.432	5.04	<.001	$\sigma^2_M = 1.74$	$\sigma^2_M = 1.75$
I within *T*	41	2.325			$\sigma^2_I = 2.80$	$\sigma^2_I = 2.79$

As with the *Ff* light reds, there is slight but appreciable variability within litters, no significant differentiation of litters within matings but significant differentiation of matings. The estimated variance components (whether litters are ignored or not) are remarkably similar to those for *Ff*. It is probable that the same array of modifiers is involved.

There was not much residual variability of the pale creams with *ee* (c^kc^k, c^kc^d or c^dc^d)*ff* judging from the visual grades of many that were produced in the earlier years of the project. Animals of genotypes *ee* (c^kc^r, c^kc^a, c^dc^r, c^dc^a)*ff* were nearly always pure white on the back although differing in the presence or absence of a tinge of very pale cream on the head.

Residual variability of pink-eyed pale sepia

Following are the analyses of variance of pale sepias of genotypes *Si*(E,e^p) *BCppFF* and the same except for *Ff*. The harmonic means of individuals per litter and per mating were 1.470 and 3.002 respectively in the former, 1.398 and 3.964 respectively in the latter. As in the other cases, *FF* and *Ff* came from strains that had been separated for several years.

Pale sepia, *Si*(E, e^p)*BCppFF*. Mean 22.79

	DF	Mean square	F	P	Variance components	
I within *L*	27	6.897			$\sigma^2_{I \cdot L} = 6.90$	
L within *M*	19	7.324	1.06	>.05	$\sigma^2_{L \cdot M} = .29$	
I within *M*	46	7.074				$\sigma^2_{I \cdot M} = 7.07$
M within *T*	13	42.564	5.81	<.001	$\sigma^2_M = 11.74$	$\sigma^2_M = 11.82$
I within *T*	59	14.893			$\sigma^2_I = 18.93$	$\sigma^2_I = 18.89$

Pale sepia, *Si(E,e^p) BCppFf*. Mean 20.39

	DF	Mean square	F	P	Variance components
I within *L*	28	10.684	1.24	>.05	
L within *M*	25	8.651			
I within *M*	53	9.725			$\sigma^2_{I \cdot M} = 9.73$
M within *T*	10	20.240	2.08	<.05	$\sigma^2_M = 2.65$
I within *T*	63	11.394			$\sigma^2_I = 12.38$

There is much more variability than in any of the other cases considered except the dingy browns. There is much variability within litters but very little or none among litters within matings in both cases. There is much differentiation among matings with *FF* but relatively little with *Ff*. There had been much more with *Ff* before separation of these strains judging from visual grades.

Space was not available for systematic genetic analysis of the large segregating differences that were apparent. No complete analysis is now possible from pedigrees since only 333 of the 1817 of these genotypes were from matings in which both parents were pale sepias, *BCppF*. Nevertheless, such analysis as can be made leaves little doubt that most of the residual variability in the late strains was due to segregation of a single pair of alleles with intermediate effect in heterozygotes. It will be convenient to designate this hypothetical pair *Mp*, *mp* (plus and minus modifiers of *pp* eumelanic colors). There is evidence, however, that in the early period, when the most extreme dilutes were appearing, there was at least one additional pair, Mp_2, mp_2.

The first row of Table 5 shows the distribution of visual grades of all nonsilvered pale sepias carrying *C* recorded from 1933 to 1954, except for those from a few matings in which there was possible confusion with lower *c* combinations, and for certain special experiments. Row 2 gives the distribution from the later strains in which *f* had been eliminated. There is still much variability but no individuals of grades 2 to 6 and only two of grade 7. A search of the earlier records revealed only one *FF* of lower grade, and this was a silvered grade 6, expected to be diluted by *sisi*. This combination was not observed otherwise except in special experiments which are not included here. The range of *Si(E,e^p) BCppFF* may be taken as very rarely exceeding visual grades 8 to 13.

Row 3 in Table 5 shows the progeny of a single rather early (1943) mating (Zp79) of the late strains (row 2). The distribution covers the range from grades 8 to 13 (mean 10.11) in spite of coming from rather high grade parents (11.12). Both parents are interpreted as *FFMpmp*. Two brother-sister matings (parental average 10.0) gave the array of row 4, with a higher average (10.85). Five matings in the next two generations (rows 5 and 6) from high grade parents (11.59) produced an array with mean 11.49 ± .10 and standard deviation

TABLE 5

Distribution of visual grades and their means among offspring of certain matings and strains

Mating	Par. mat.	Colors and grades of parents: male	female	$\bar{P}$	Genotypes of parents: male	female	Visual grades of offspring: 2	3	4	5	6	7	8	9	10	11	12	13	14	No.	Mean
1. (1933–54)	Total						5	6	14	41	61	103	219	330	479	338	197	21	3	1817	9.51
2. Late strains					*FF*	*FF*	..	..	..	..	..	2	20	72	190	128	85	8	..	505	10.40
3. Zp79	.....	S_{11}	S_{12}	11.5	*FFMpmp*	*FFMpmp*	..	..	..	..	..	..	3	2	7	5	1	1	.	19	10.11
4. Zp107,108	(3)(3)	$S_{10,11}$	$S_{10,9}$	10.0	*MpMp*	*Mpmp*	..	..	..	..	..	..	..	5	4	2	7	2	..	20	10.85
5. Zp118,119,123	(4)(4)	$S_{10,11,11}$	$S_{13,9,11}$	11.3	*MpMp*	*MpMp*	..	..	..	..	..	..	..	..	3	16	14	3	..	36	11.47
6. Zp126,137	(5)(5)	$S_{13,13}$	$S_{11,12}$	12.2	*MpMp*	*MpMp*	..	..	..	..	..	..	..	..	..	8	9	..	..	17	11.53
7. Zpb65	.....	S_{12}	Br_9		*FFMpmp*	*FFMpmp*	..	..	..	..	..	1	2	..	3	1	1	..	..	8	9.50
8. Zpb69	(7)(7)	S_{11}	S_7		*MpMp*	*mpmp*	..	..	..	..	..	..	..	..	6	..	..	..	..	6	10.00
9. Zpb79.80	(8)(8)	$S_{10}Br_9$	S_{10}		*Mpmp*	*Mpmp*	..	..	..	..	..	1	5	2	14	1	1	..	..	24	9.50
10. (1933–54)	Total				*F–*	*ff*	..	2	3	10	13	33	70	85	66	25	10	..	..	317	8.70
11. Late strains					*F–*	*ff*	..	..	..	3	1	5	39	36	31	19	8	..		142	9.26
12. Zfp268	.....	S_{12}	pCr		*FFMpmp*	*ffMpmp*	..	..	..	2	1	..	6	1	7	4	.	..	..	21	8.90
13. Zfp286	.....	pCr	S_{10}		*ffMpmp*	*FfMpMp*	..	..	..	..	.	..	1	6	2	1	..	..	..	10	9.30
14. Zfp302	.....	pCr	S_{10}		*ffMpMp*	*FfMpMp*	..	..	..	..	..	..	..	1	4	3	1	..	..	9	10.44
15. RP60	.....	S_9	pCr		*FfMpMp*	*ffMpMp*	..	..	..	..	..	..	..	3	5	5	1	..	..	14	10.29
16. EA113	.....	S_4	S_8		*Ffmpmp*	*FFmpmp*	3	2	1	..	..	2	2	..	..	..	..	..	..	10	4.60
17. ZpCa4	(16)..	S_8	W		*FFmpmp*	*Ffmpmp*	1	..	..	3	..	1	..	..	..	..	..	..	..	5	4.80
18. ZpCa1	(16)..	S_8	W		*FFmpmp*	*FfMpmp*	..	1	1	..	..	..	2	1	..	1	..	..	..	6	7.17
19. ED172	..(16)	Y_8(P)	S_4		*FFMpMp*	*Ffmpmp*	..	..	..	3	3	..	1	..	..	..	..	..	..	7	5.86
20. ED173	..(16)	Y_8(P)	S_7		*FFMpMp*	*FFmpmp*	..	..	..	..	..	..	..	..	1	1	..	..	..	2	10.50
21. Zpb1	.....	S_5	S_5		*Ffmpmp*	*Ffmpmp*	..	..	1	2	2	..	..	..	..	..	..	..	..	5	5.20
22. Zfp6	(21)..	pCr	W(P)		*ffmpmp*	*FFMpmp*	..	2	1	2	..	1	1	..	..	..	..	..	..	7	5.00
23. Zfp11	(22)(22)	S_3	S_3		*Ffmpmp*	*Ffmpmp*	1	..	1	..	2	1	..	1	2	..	..	..	..	8	6.75

The parental matings (par. mat.) of sire and dam are indicated if in the table. The colors (S=pale sepia, Br=pale brown, pCr=pale brownish cream, W= albino, Y=yellow) and visual grades of sire and dam and in some cases the averages are shown. The interpretation of the genotypes of the sire and dam are given. Reciprocal matings are included in rows 10,11.

.70 ± .07. These may be taken as representative of *FFMpMp* on the hypothesis above.

While some lines such as the above rapidly reached a high average and low variability, a low average and high variability persisted in other cases. Row 7 shows the progeny of a late mating (1949) which produced one of the two of grade 7 in row 2. Like Zp79 of row 3 both parents, though rather intense for pale sepia (12) and for pale brown (9), are interpreted as *FFMpmp*. A brother-sister mating between offspring that were almost at opposite extremes (grades 11 and 7) produced a progeny that was uniformly sepia grade 10 except for two pale browns of grades 8 and 9. A mating of type *MpMp* × *mpmp* is suggested. The wide range, including the only other individual of grade 7 in row 2, of the offspring from two brother-sister matings (interpreted as *Mpmp* × *Mpmp*) are in harmony (row 9).

These matings in row 9 were producing young in the period in which reflectionmeter readings were being taken. Indices were obtained for 68 individuals from 23 matings of the group in which *f* had been eliminated. These matings were apportioned into three groups, interpreted as *Mpmp* × *Mpmp*, *MpMp* × *Mpmp* and *MpMp* × *MpMp* on the basis of parentage and the lowest visual grade or reflectionmeter index among their offspring. The distribution of the grades and indices are shown in Table 6. Rough estimates of the means for the various genotypes can be obtained by dividing the progeny of those called *Mpmp* × *Mpmp* as closely as possible on 1:2:1 basis, of those called *MpMp* × *Mpmp* on a 1:1 basis and treating all progeny of those called *MpMp* × *MpMp* as of this genotype. This leads to the averages shown in Table 7. The mean indices have been transformed into estimated visual grades and both visual grades and indices into relative amounts of pigment. There is fair agreement. It appears that *FFmpmp* has about half as much pigment as *FFMpMp* with the heterozygotes about half way between.

These estimates, which assume a minimum amount of overlap should tend to exaggerate the differential effects. The pedigree analysis indicates, however, that these are approximately correct.

We consider next the effects of *Mp*, *mp* in individuals known to be *Ff* because they were produced by a mating of type *F-* × *ff*. The distribution of all such individuals produced from 1933–1954 is shown in row 10 of Table 5. The upper limit reaches grade 12, only one grade less than for known *FF* (row 2), but the lower limit extends three grades lower than for known *FF*. There are 127 *Cpp* sepias of grade 6 or less in row 1. These include one *sisiFF* of grade 6 already referred to and 28 known to be *Ff* in row 10. All of the rest could have been *Ff* as far as ancestry is concerned. One or both parents of half of them were known to be *Ff*. In the other half, ancestry had to be traced one or two or, for two individuals, three generations to encounter known *Ff*. All of them were produced by six more or less connected small clusters, characterized by segregation of both *ff* and the low grade pale sepias. Moreover, among the 103 *Cpp* sepias of grade 7 in row 1,

TABLE 6

Distributions of visual grades and reflectionmeter indices of young from matings in which latter were taken. Matings classified primarily according to whether FF × FF *or* F– × f f *(reciprocally) and secondarily according to whether interpreted as* MpMp × MpMp, MpMp × Mpmp *(reciprocally) or* Mpmp × Mpmp *on the basis of distribution of grades and indices as described in the text*

Parental genotypes		No. mat.	Visual grade										Reflectionmeter index											
			5	6	7	8	9	10	11	12	No.	Mean	12–	14–	16–	18–	20–	22–	24–	26–	28–	30–	No.	Mean
FF × *FF*	*MpMp* × *MpMp*	7	..	..	..	..	..	3	7	6	16	11.19	..	..	..	..	..	..	5	4	..	..	9	25.7
FF × *FF*	*MpMp* × *Mpmp*	9	..	..	..	..	2	14	23	7	46	10.76	..	..	..	1	3	9	5	3	1	1	23	24.2
FF × *FF*	*Mpmp* × *Mpmp*	7	..	..	1	8	13	42	13	9	86	9.99	..	1	6	7	7	5	6	..	3	1	36	21.6
F– × *ff*	*MpMp* × *MpMp*	4	..	..	..	..	3	6	7	1	17	10.35	..	..	..	1	5	5	3	1	..	..	15	22.8
F– × *ff*	*MpMp* × *Mpmp*	8	..	..	2	13	16	11	6	3	51	9.29	..	1	9	12	5	5	5	..	..	..	37	19.9
F– × *ff*	*Mpmp* × *Mpmp*	2	3	1	.	6	2	8	4	..	24	8.79	2	2	1	1	4	..	2	1	..	..	13	19.1

TABLE 7

Mean grades of genotypes as deduced from Table 6 and relative amounts of pigment (intense black=100) as estimated by transformation of mean grades, mean indices of genotypes as deduced from Table 6 and corresponding grades and amounts of pigment as estimated by transformation of the mean indices

Genotype	No.	Mean grade	Est. pigment	No.	Mean index	Estimated Grade	Estimated Pigment
FF MpMp	61	11.3	23.6	29	26.2	12.0	26.8
Mpmp	65	10.1	19.2	30	21.6	9.9	18.6
mpmp	21	8.5	14.3	9	16.9	7.8	12.3
Ff MpMp	50	10.3	19.9	36	22.7	10.4	20.4
Mpmp	38	8.5	14.3	25	18.1	8.3	13.8
mpmp	4	5.3	7.0	4	13.7	6.3	9.0

all but six were from these same clusters. It is probable that virtually all of grades 6 or less and most of grade 7 were *Ff*.

There were only four of grades six or less, however, among the 142 *CppFf* sepias of the later strains in which *f* was carried (row 11). Three of these came from one mating, Zfp268, the distribution of which is given in row 12. In spite of the high grade of the male (12) this is interpreted as *FFMpmp* × *ffMpmp*. Row 13 gives the distribution of a more typical mating of this group, interpreted as probably *ffMpmp* × *FfMpMp*. Mating Zfp302, row 14, gave the highest average grade for *Ff* (10.44) among matings with nine or more offspring. The array in row 15 from an unrelated series is closely similar (10.29). These two matings may be taken as representative of *SiEBCppFfMpMp* (mean 10.35 ± .18, standard deviation .88 ± .13) slightly more than a grade lower than for *FFMpMp*.

The matings in rows 12, 13 and 14 all produced young from which reflectionmeter indices were obtained. Altogether 65 indices of individuals known to be *SiEBCppFf* were obtained from 14 matings of type *F-* × *ff* (92 visual grades). These matings were apportioned and mean grades and indices for genotypes were obtained in the same way as for matings of type *FF* × *FF*. There is fairly good agreement between the mean visual grades estimated from the mean indices and those estimated directly. In this case *mpmp* appears to produce somewhat less than half as much pigment as *MpMp*, with *Mpmp* again intermediate.

None of the very palest *Cpp* sepias (grades 2 to 4) are included in the group considered above (row 11). Row 16 gives the array from mating EA113 which produced more than any other for which grades are available. The father was a very pale sepia, born before visual grades were taken, but probably of grade 3 or 4 from his description. The female was of intermediate intensity probably 7 or 8. Both came from the cluster that was the source of nearly all extremely dilute *pp* sepias. Both parents could have been *Ff*, but, as no *ff* young were produced, the mating is interpreted as *Ffmpmp* × *FFmpmp* with segregation of *Ffmpmp* and *FFmpmp*, the former especially at a decidedly lower level than estimated above. A relatively intense son, grade 8, (presumably *FFmpmp*) was mated with two

albinos from the same cluster (rows 17 and 18). They both produced very low grade young, indicating transmission of *f* and *mp*, but, while one produced no young more intense than grade 7 (probably *FFmpmp*), the other produced young as intense as grades 9 and 11 (interpreted as *FFMpmp*). Two other offspring of EA113 (grade 4, interpreted as *Ffmpmp*, row 19 and grade 7, interpreted as *FFmpmp*, row 20) were mated with an unrelated yellow male (eec^dc^dpp). One produced young ranging from grades 5 to 8, the lighter of which indicate *Ffmpmp* in terms of the estimates from the later matings but which may be exceptionally light *FfMpmp*. The other produced only two rather intense offspring, grades 9 and 11, presumably *FFMpmp*. The father of both progenies was thus probably *FFMpMp*.

Rows 21 to 23 show a chain of matings from another remotely related cluster that produced some exceptionally pale *Cpp* sepias. Both parents in Zpb-1 (brother and sister both grade 5) were *Ff* and are interpreted as *mpmp*. They seem to have produced no *FF* segregants. A mating of a pale brownish cream offspring (*ff*) with an unrelated albino (c^ac^aFF) produced seven pale sepias ranging from grades 3 to 8 (segregates of *Ffmpmp* and *FfMpmp*). A mating between the two palest of these (both grade 3 and presumably *Ffmpmp*) produced an extraordinary range of intensities, grades 2 to 10. More than segregation of *Ff* and *FF* seems required.

The extremely pale *Cpp* sepias from these early clusters illustrated in rows 16 to 23 and the wide segregation in some cases seem to require an additional pair of alleles Mp_2, mp_2 with differential effects comparable to those of the first pair (which may now be called Mp_1, mp_1) and cumulative with them and with *F*, *f*.

The same modifiers that affect *Cpp* sepias clearly affected those with lower *c* alleles even more on a percentage basis, but analysis is complicated by the effects of *Dm,dm* and less frequently *Si,si*. Pink-eyed pure whites and pale yellows, demonstrated by breedings tests to be EBc^dc^appF appeared in the early clusters in which the palest *Cpp* sepias were found. These may be interpreted as having fallen below the threshold for any eumelanic pigmentation from the effect of the minus modifiers mp_1 and mp_2. Whether underlying yellow pigment is revealed or not may be interpreted as depending on whether segregation of other factors such as *Dm,dm* and *Si,si* put the yellow forming process above or below its threshold.

Residual variability of pink-eyed pale brown

The analysis of variance of the indices of pale browns of genotype $Si(E,e^p)$ *bbCppF* is given below, again with separation of *FF* and *Ff* which came from the two separate groups of strains referred to in connection with the yellows and the pale sepias. The harmonic means for individuals per litter and per mating were 1.462 and 3.586 respectively for *FF* and 1.321 and 3.708 respectively for *Ff*.

The analyses are rather similar. There is appreciable variability within litters but so much variability of litters within matings that differentiation of matings is not significant, or doubtfully so. There is undoubtedly residual genetic variability but no factorial analysis is practicable in these data.

Pink-eyed pale brown, *Si(E,e^p)bbCppFF*. Mean 19.93

	DF	Mean square	F	P	Variance components
I within *L*	17	.919			$\sigma^2_{I\cdot L} = .92$
L within *M*	10	5.079	5.52	<.01	$\sigma^2_{L\cdot M} = 2.85$
I within *M*	27	2.460			
M within *T*	8	12.292	2.42	>.05	$\sigma^2_M = 2.01$
I within *T*	35	4.709			$\sigma^2_I = 5.78$

Pink-eyed pale brown, *Si(E,e^p)bbCppFf*. Mean 18.40

	DF	Mean square	F	P	Variance components
I within *L*	21	2.161			$\sigma^2_{I\cdot L} = 2.16$
L within *M*	25	8.046	3.72	<.001	$\sigma^2_{L\cdot M} = 4.45$
I within *M*	46	5.360			
M within *T*	10	18.026	2.24	ca .05	$\sigma^2_M = 2.69$
I within *T*	56	7.622			$\sigma^2_I = 9.90$

In the pedigree analysis of the early records, it was, however, apparent that exceptionally light *Cpp* browns (visual grade 6 or less) occurred only in the same clusters and often segregated from the same matings as those which produced the exceptionally pale *Cpp* sepias. There is no doubt that the same modifiers of type *Mp,mp* acted similarly in the pale browns and pale sepias though the differential effects seem to have been somewhat less in the pale browns.

It may be noted here that these same clusters and matings also produced genotypes of the other major series. Tabulations gave no indication of any associated effect of these modifiers on dark-eyed sepias, dark-eyed browns or yellows.

Residual variability of pale brownish creams

The analysis of variance of the indices of animals of genotype *ECppff* (in which *B* and *bb* cause no perceptible difference) gave the following results. The harmonic means of individuals per litter and per mating were 1.367 and 3.338, respectively.

The differentiation of litters within matings is not significant in spite of relatively large numbers. There is considerable differentiation within litters and a small but significant amount of differentiation of matings.

It is obvious from inspection of the animals that there is residual variability in both the very small amount of eumelanin and the larger amount of phaeomelanin. This genotype was originally described as pure pale yellow indistinguish-

Pale brownish cream, *ECppff*. Mean 4.93

	DF	Mean square	F	P	Variance components
I within *L*	42	2.229			$\sigma^2_{I\cdot L} = 2.33$
L within *M*	46	3.040	1.36	>.05	$\sigma^2_{L\cdot M} = .59$
I within *M*	88	2.653			$\sigma^2_{I\cdot M} = 2.65$
M within *T*	26	6.766	2.23	<.01	$\sigma^2_M = 1.12$ $\sigma^2_M = 1.23$
I within *T*	114	3.591			$\sigma^2_I = 3.94$ $\sigma^2_I = 3.88$

able from *eec^{d}c^{d}ff* even on direct comparison of live animals (at birth) (WRIGHT 1927). The original strain was discontinued. When reconstituted many years later, the animals usually showed a slight brownish tinge that was unmistakable on comparison with *eec^{d}c^{d}ff*. As the original strain was closely related to those that gave rise to abnormally pale *Cpp* sepias, while the later one was developed after selection of the latter for higher intensity, it is probable that minus modifiers of *pp*-eumelanin (mp_1, mp_2) prevailed in the earlier strain, the plus alleles (Mp_1, Mp_2) in the later one. Mp_1 and Mp_2 may be considered as feeble substitutes for *F*, itself a feeble substitute for *P* in the production of eumelanin.

There was another difference, however, in that e^p prevailed in the earlier strain, *E* in the later one, and it has been apparent that the pale cream (in the pale cream-yellow tortoise-shells *e^{p}e^{p}Cppff* that were produced in small numbers in the later strain) was of a somewhat purer cream than the self-colored brownish creams *ECppff*.

The variations in the intensity of the phaeomelanic component probably depended on *Dm,dm* and perhaps *Si,si* and on factors that affect the threshold for yellow.

Summary

The present paper is concerned with the residual variability in the intensities of the various major color varieties in a large crossbred but self-contained guinea pig colony. It is largely restricted to the genotypes carrying the highest allele (*C*) of the albino series. Analyses of variance of reflectionmeter indices are presented in each of 11 cases.

The most interesting results are those with dark-eyed dark browns (*EbbCPF*). There is slight but significant variability due to factors $\Sigma(Lb)$ that are interpreted as affecting the limiting amount of brown pigment. Enormously more variability in certain strains is interpreted as due to excess product of the genes *C*, *P* and *F* concerned with tyrosinase. The combination *c^{k}c^{k}ppff* is pure white. Addition of *C*, *F* or *P* permits production of brown pigment ranging from a trace to full intensity. Maximum observed intensity is with *CPpff*. Combinations *CPpF*, *CPPff* and *CCPPFF* are progressively lighter. The last may be as pale as

CppF except at the tip of the hair. With sufficient plus modifiers, however, *CCPPFF* can be of full intensity. The spotting factor (*s*) produces mosaicism with respect to this effect in the brown parts of the coat of dilute (dingy) *CCPPFF*, related developmentally to the pattern of brown and white.

Next in importance are wide variations in the intensity of pale sepias (*EBppF*). These are shown to depend on modifiers tentatively called Mp_1, mp_1, Mp_2, mp_2 interpreted as affecting the intensity of eumelanin, but appreciable only in the absence of *P*. They also affect pale brown (*EbbppF*) and probably pale brownish cream (*ECppff*) in which the plus alleles, Mp_1, Mp_2, are interpreted as functioning as very feeble substitutes for *F*, itself a feeble substitute for *P* as a cofactor of the *c* alleles in producing eumelanin.

There was residual genetic variability in other cases, but it was too slight for even tentative analysis in terms of loci.

The relations of the major factors and arrays of modifiers (identified in the text) to the processes that seem to be indicated are illustrated in Figures 2 and 3.

LITERATURE CITED

CASTLE, W. E., 1929 A mosaic (intense-dilute) coat pattern in the rabbit. J. Exptl. Zool. **52**: 471–479.

CASTLE, W. E., and S. WRIGHT, 1916 Studies of inheritance in guinea pigs and rats. Carnegie Inst. Wash. Publ. **241.**

CHASE, H. B., 1939 Studies on the tricolor pattern of the guinea pig. II. The distribution of black and yellow as affected by white spotting and by imperfect dominance in the tortoise-shell series of alleles. Genetics **24**: 610–643.

DETLEFSEN, J. A., 1914 Genetic studies on a cavy species cross. Carnegie Inst. Wash. Publ. **205.**

DUNN, L. C., 1934 Analysis of a case of mosaicism in the house mouse. J. Genet. **29**: 317–326.

FOSTER, M., 1956 Enzymatic studies of the physiological genetics of guinea pig coat coloration. I. Oxygen consumption studies. Genetics **41**: 396–409.

GINSBURG, B., 1944 The effect of the major genes controlling coat colors in the guinea pig on the dopaoxidase activity of skin extracts. Genetics **29**: 176–198.

HEIDENTHAL, G., 1940 A colorimetric study of genic effect on guinea pig color. Genetics **25**: 197–214.

IBSEN, H. L., 1932 Modifying factors in guinea pigs. Proc. 6th Intern. Congr. Genet. **2**: 97–101.

IBSEN, H. L., and B. L. GOERTZEN, 1951 Whitish, a modifier of chocolate and black hairs in guinea pigs. J. Heredity **42**: 231–236.

ILJIN, N. A., 1928 The distribution and inheritance of white spots in guinea pigs (Russian). Trans. Lab. Exptl. Biol. Zoo-Park Moscow **4**: 255–349.

RUSSELL, E. S., 1939 A quantitative study of genic effects on guinea pig coat colors. Genetics **24**: 332–355.

RUSSELL, W. L., 1939 Investigation of physiological genetics of hair and skin color in the guinea pig by means of the dopa reaction. Genetics **24**: 645–667.

WOLFF, G. L., 1955 The effect of environmental temperature on coat color in diverse genotypes of the guinea pig. Genetics **40**: 90–106.

WRIGHT, S., 1917 Color inheritance in mammals. V. The guinea pig. J. Heredity **8**: 476–480.
1923 The relation between the piebald and tortoise-shell color patterns in guinea pigs. Anat. Record **23**: 393.
1927 The effects in combination of the major color factors of the guinea pig. Genetics **12**: 530–569.
1947 On the genetics of several types of silvering in the guinea pig. Genetics **32**: 115–141.
1949 Estimates of amounts of melanin in the hair of diverse genotypes of the guinea pig from transformation of empirical grades. Genetics **34**: 245–271.
1959a Silvering (*si*) and diminution (*dm*) of coat color of the guinea pig, and male sterility of the white or near-white combination of these. Genetics **44**: 563–590.
1959b A quantitative study of variations in intensity of genotypes of the guinea pig at birth. Genetics **44**: 1001–1026.

WRIGHT, S., and Z. I. BRADDOCK, 1949 Colorimetric determination of the amounts of melanin in the hair of diverse genotypes of the guinea pig. Genetics **34**: 223–241.

RECIPROCAL CROSSES IN STREPTOMYCES COELICOLOR[1]

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THE interaction of two complementary growth-factor dependent mutants, i.e., complementary auxotrophs, of *Streptomyces coelicolor* to form growth-factor independent progeny, i.e., prototrophs, was interpreted as gene recombination by SERMONTI and SPADA-SERMONTI (1955) and by BRAENDLE and SZYBALSKI (1959), but as heterokaryosis by BRADLEY (1957). The former workers concluded that gene recombination was operative because (1) nonparental auxotrophs were also isolated from mixed cultures of the two parental types and (2) all of the recombinant classes tested were found to be stable. Conversely, BRADLEY (1958a) obtained infrequent dissociates regularly from nonparental auxotrophic recombinants and from prototrophic recombinants; only parental dissociates were stable. Recently SERMONTI and SPADA-SERMONTI (1959) derived diverse types from recombinant clones.

In order to elucidate the recombinational process, BRAENDLE and SZYBALSKI (1957) carried out "reciprocal crosses" using streptomycin susceptibility as an unselected marker. They found that when streptomycin resistance was coupled with proline and glutamate independence, and streptomycin sensitivity was coupled with methionine and histidine independence, all of the recombinants were drug resistant. Contrariwise, when streptomycin resistance was coupled with methionine and histidine independence, and streptomycin sensitivity with proline and glutamate independence, almost all of the recombinants were inhibited by streptomycin. Although this correlation could have resulted from gene recombination, interpretations based on the data of BRAENDLE and SZYBALSKI were inconclusive because progeny arising from the cross involving the two streptomycin resistant parental strains were not analyzed.

This investigation was concerned with reciprocal crosses using streptomycin susceptibility and actinophage susceptibility as unselected markers. The progeny of sensitive by sensitive crosses and of sensitive by resistant crosses were sensitive. Conversely, the progeny of resistant by resistant crosses were resistant. These results prove that prototrophs derived from mixed cultures of complementary auxotrophs are not simple haplophase recombinants. These findings are consistent with the hypotheses that these prototrophs are heterokaryons or heterozygous diploids.

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MATERIALS AND METHODS

The strains used in this study were mutants derived from *S. coelicolor* strain WAc-199 and *S. coelicolor* strain 1 (Table 1). The former wild type strain was isolated from a Wisconsin soil sample whereas the latter wild type strain, and two mutant derivatives, 5 and 23, were generously provided by DR. G. SERMONTI, Istituto Superiore di Sanita, Rome. The epithet, *S. coelicolor*, has been retained here even though DR. H. KUTZNER and DR. S. WAKSMAN, Rutgers University, suggested that these organisms should be designated *S. violaceoruber* (private communication).

Stock cultures were grown at 30°C on complete medium of the following composition: glucose, 20 gms; agar (Difco), 15 gms; yeast extract (Difco), 1 gm; casitone (Difco), 1 gm; $MgSO_4 \cdot 7H_2O$, 0.5 gm; KNO_3, 2 gms; K_2HPO_4, 2 gms; deionized water, 1 L. The salts of nitrate and phosphate were autoclaved at 121°C for 12 minutes as one double-strength solution, and the remaining components as the second double-strength solution. The two aliquots were mixed immediately before dispensing into petri plates. Minimal medium was made by deleting yeast extract and casitone from the complete medium formula. Growth-factor supplements, i.e., amino acids and uracil, were added to the minimal medium, when indicated, at a concentration of 10 mg/L.

Auxotrophic mutants were obtained by indirect selection subsequent to treatment with ultraviolet irradiation (BRADLEY and LEDERBERG 1956). Streptomycin resistant variants and actinophage resistant variants were obtained by direct selection (BRADLEY 1958a). Streptomycin resistant mutants grew well on complete medium to which 25 mg/L dihydrostreptomycin sulfate had been added. Actinophage susceptibility was determined on peptone-yeast extract medium by cross brushing the organism to be tested against a three cm streak of 10^7 to 10^8 phage particles (BRADLEY 1959a). Sensitive streptomycetes were lysed at the phage-host

TABLE 1

Description of principal cultures

Strain	Ancestor	Phenotype*
1	wild type from G. SERMONTI, Rome	prototrophic, blue V^s S^s
5	1	met-his-blue V^s S^s
23	5	met-his-blue V^s S^r
199	wild type from Wisconsin soil sample	prototrophic, blue V^s S^s
202	199	cys-pro-pink V^s S^s
203	199	arg-ura-blue V^s S^s
209	202	cys-pro-pink V^r S^s
210	203	arg-ura-blue V^r S^s
214	203	arg-ura-blue V^s S^r

* V^s/V^r: sensitive/resistant to actinophage MSP-10; S^s/S^r: sensitive/resistant to 25 mg/L dihydrostreptomycin sulfate; met-, his-, cys-, pro-, arg-, and ura-: required added methionine, histidine, cystine, proline, arginine, and uracil respectively.

intersection whereas resistant streptomycetes grew uninterrupted across the phage streak.

Recombinants were isolated by the following procedure: colonies from two auxotrophic strains, grown separately on complete medium, were cut from the agar and minced; sufficient fragmented mycelium of each parent was plated together on complete agar plates to yield confluent growth; after 4–6 days at 30°C, a sample of the mixed growth was transferred to minimal medium agar by replica plating (LEDERBERG and LEDERBERG 1952); colonies that developed on minimal medium after 4–6 days were purified by three serial transfers on complete medium; prototrophy was verified by replating the purified recombinants to minimal medium.

RESULTS

Streptomycin susceptibility as an unselected marker

The progeny resulting from crosses involving two streptomycin sensitive parents ($S^s \times S^s$) were invariably streptomycin sensitive (Table 2). Conversely, the recombinant progeny of two streptomycin resistant parents were invariably streptomycin resistant. Prototrophic recombinants obtained from parents differing with respect to streptomycin susceptibility were completely inhibited by 25 mg/L dihydrostreptomycin sulfate, or yielded a few vigorously growing colonies (Figure 1). From the cross of strain 23 by strain 210 (met-his-blue $S^r \times$ arg-ura-blue S^s) 365 recombinant colonies were inhibited completely by streptomycin,

TABLE 2

Results of reciprocal crosses employing streptomycin susceptibility and actinophage susceptibility as unselected markers

Strains crossed	Unselected markers	Phenotypes of purified recombinants			
		V^sS^s	V^rS^s	V^sS^r	V^rS^r
5 × 210	$S^s \times S^s$	283	0	0	0
23 × 214	$S^r \times S^r$	0	0	469	0
5 × 214	$S^s \times S^r$	215 + 175*	0	0	0
23 × 210	$S^r \times S^s$	365 + 136*	12	1	0
202 × 203	$V^s \times V^s$	344	1	0	0
209 × 210	$V^r \times V^r$	0	373	0	0
202 × 210	$V^s \times V^r$	197 + 166†	0	0	0
209 × 203	$V^r \times V^s$	534	19	0	0

Complementary auxotrophs were grown together on complete medium for 4–6 days; prototrophic recombinants were selected by replica plating to minimal medium; each recombinant colony was purified by three serial subcultures on complete medium; prototrophy was verified by replating to minimal medium.

* A few S^r colonies developed (less than ten percent of the inoculum).

† Scant growth at the phage-host intersection.

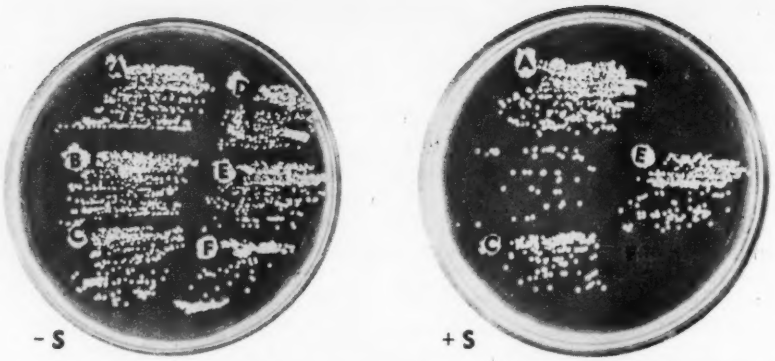

FIGURE 1.—Assay for susceptibility to streptomycin. Strain 23 (parental S^r), designated as A, grew equally well on complete medium (—S) and medium containing 25 mg/L dihydrostreptomycin sulfate (+S). Strain 210 (parental S^s), designated as F, grew on complete medium only. B is an incompletely susceptible recombinant; C and E, which are from the same recombinant colony, are totally resistant; D is a completely susceptible recombinant.

but 1–10 percent of the inoculum from 136 recombinant colonies grew on complete medium containing streptomycin. Only one colony consisted solely of streptomycin resistant plating units. From the cross of strain 5 by strain 214 (met-his-blue S^s × arg-ura-blue S^r) 215 streptomycin sensitive colonies and 175 incompletely susceptible colonies were found.

During serial replating of prototrophs, whether drug sensitive or resistant, parental types were recovered. The variety of dissociates, their frequency and stability, confirmed previous findings (BRADLEY 1958b).

Actinophage susceptibility as an unselected marker

According to expectation, progeny from crosses involving two actinophage sensitive parents were sensitive. Corresponding crosses between two actinophage resistant parents yielded virus resistant recombinants. Prototrophic recombinants obtained from parents differing with respect to phage susceptibility were usually inhibited completely by virus, or were slightly resistant. Rarely recombinants were fully resistant to actinophage. From crosses of strain 209 by strain 203 (cys-pro-pink V^r × arg-ura-blue V^s), 534 recombinants were found to be totally sensitive whereas 19 were entirely resistant to virus. In the reciprocal cross, i.e., strain 210 by strain 202 (arg-ura-blue V^r × cys-pro-pink V^s), 197 of the purified prototrophs were very susceptible to actinophage, but 166 grew scantily with an excess of phage (Figure 2). As before, recombinant colonies gave rise to parental dissociates infrequently.

DISCUSSION

Stable prototrophic progeny can arise from mixed cultures of two complementary auxotrophs as a result of (1) mutation, either spontaneous or induced,

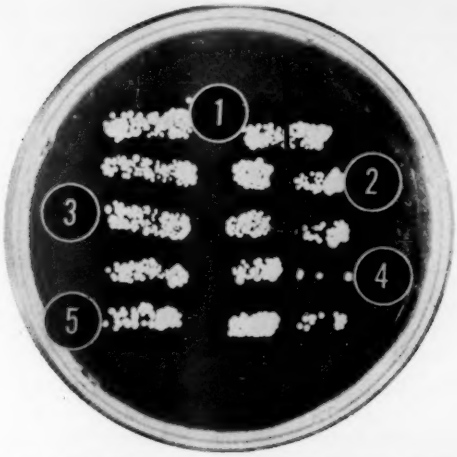

FIGURE 2.—Assay for susceptibility to actinophage. The arrow indicates the site of viral inoculum. Cross streak No. 1 is strain 210 (parental V^r); No. 2 is strain 202 (parental V^s); No. 3 is an incompletely susceptible recombinant; No. 4 and No. 5 are very susceptible recombinants.

to nutritional independence; (2) fusion of haploid nuclei to form diploid nuclei, which segregate immediately to form haploid recombinant nuclei; (3) fusion of haploid nuclei to form stable diploid nuclei; (4) gene recombination between diploid nuclei; (5) establishment of heterokaryosis which is perpetuated through heterokaryotic spores; (6) interactions between part of a nucleus and an intact nucleus to form a partially diploid nucleus that may or may not be stable. Spontaneous mutation is not responsible for the origin of the observed prototrophs because the rate of prototroph formation in two membered cultures exceeds greatly the rate at which the multiple marked parents singly revert to the wild type. Culture filtrates of individual parents or of mixed cultures do not increase significantly the rate of reversion; therefore the induction of mutations to prototrophy by products of one parent or products from interactions between the parents is improbable. Inasmuch as cell contact is necessary for prototroph formation, actinophage mediated transduction and nucleic acid mediated transformation are not responsible for the origin of the recombinants. At the present time, too few characteristics have been studied to rule out merozygosis (hypothesis No. 6) conclusively. Recovery of novel phenotypes and parental types from recombinants is inconsistent with simple haplophase gene recombination; however the possibility exists that these dissociates or segregants represent a unique fraction of the population or are an artifact resulting from unintentionally selective media and subjective selective biases.

SERMONTI and SPADA-SERMONTI (1956) noted that even on partially supplemented media, prototrophic recombinants constituted an overwhelming proportion of the recombinant population. BRADLEY and ANDERSON (1958) and BRAD-

LEY, ANDERSON and JONES (1959) found that when actinophage susceptibility or streptomycin susceptibility was used as an unselected marker, all of the recombinants were sensitive. If haplophase recombination were operative, these findings would be interpreted in terms of linkage. The present investigation demonstrated that in crosses involving a streptomycin sensitive parent and a resistant parent, the recombinants were antibiotic sensitive regardless of how the nutritional requirements and drug susceptibility were coupled in the parents. Similar results were obtained with respect to actinophage susceptibility.

These results are contrary to those reported by BRAENDLE and SZYBALSKI (1957) who reported that streptomycin susceptibility seemed to be linked very strongly to glutamic acid. Inasmuch as BRAENDLE and SZYBALSKI did not cross the two streptomycin resistant strains, it is not established that the two resistant strains were the result of identical mutational events. Moreover, streptomycin sensitivity was assumed to be invariably dominant to resistance but no supporting data were provided. The crosses reported by these workers therefore are not valid reciprocal crosses.

It must be emphasized that even though sensitive by sensitive and resistant by resistant crosses were performed in this investigation, the sameness of the mutations for resistance has not been unquestionably established. These results do show that simple haplophase gene recombination is not the principal genetic process in this system. These data do not differentiate between stable diploidy and stable heterokaryosis. The consistent recovery of parental dissociates and dissociates having novel phenotypes, and the variable expressivity of unselected markers is indicative of a complex series of genetic interactions (BRADLEY 1959b).

HOPWOOD (1959) working with another strain of *S. coelicolor* has found complementary recombinant phenotypes in equal numbers among the progeny of crosses. The discrepancies between his results and the results reported here may be a manifestation of strain differences. As HOPWOOD has pointed out, *S. coelicolor* is in fact a heterogeneous group of organisms. Genuine strain-to-strain differences in behavior, therefore, are not unexpected.

SUMMARY

Complementary, growth-factor dependent, mutant pairs of *Streptomyces coelicolor* interacted to form growth-factor independent recombinants. When both parents were streptomycin sensitive or actinophage sensitive, the selected prototrophic recombinants were sensitive. Similarly, when both parents were drug resistant or virus resistant, the recombinant progeny were resistant. When the parents differed with respect to susceptibility to streptomycin or actinophage, recombinants were sensitive, irrespective of the coupling in the parental strains. Failure to recover both unselected phenotypes in reciprocal crosses proved that haplophase gene recombination was not the principal process operative. These results are consistent with the hypotheses that the recombinants are heterokaryons or heterozygous diploids.

LITERATURE CITED

BRADLEY, S. G., 1957 Heterokaryosis in *Streptomyces coelicolor*. J. Bacteriol. **73**: 581–582.

1958a Genetic analysis of segregants from heterokaryons of *Streptomyces coelicolor*. J. Bacteriol. **76**: 464–470.

1958b Genetics of antibiotic-producing streptomycetes. Univ. Minn. Med. Bull. **30**: 146–154.

1959a Cross resistance patterns of mutants resistant to streptomyces—phages. Mycologia **51**: 125–131.

1959b Mechanisms controlling variation in streptomycetes. Annals. N.Y. Acad. Sci. **81**: 899–906.

BRADLEY, S. G., and D. L. ANDERSON, 1958 Compatibility system controlling heterokaryon formation in *Streptomyces coelicolor*. Proc. Soc. Exptl. Biol. Med. **99**: 476–478.

BRADLEY, S. G., D. L. ANDERSON, and L. A. JONES, 1959 Genetic interactions within heterokaryons of streptomycetes. Annals. N.Y. Acad. Sci. **81**: 811–823.

BRADLEY, S. G., and J. LEDERBERG, 1956 Heterokaryosis in Streptomyces. J. Bacteriol. **72**: 219–225.

BRAENDLE, D. H., and W. SZYBALSKI, 1957 Genetic interaction among streptomycetes: heterokaryosis and synkaryosis. Proc. Natl. Acad. Sci. U.S. **43**: 947–955.

1959 Heterokaryotic compatibility, metabolic cooperation, and genic recombination in Streptomyces. Annals. N.Y. Acad. Sci. **81**: 824–851.

HOPWOOD, D. A., 1959 Linkage and the mechanism of recombination in *Streptomyces coelicolor*. Annals. N.Y. Acad. Sci. **81**: 887–898.

LEDERBERG, J., and E. M. LEDERBERG, 1952 Replica plating and indirect selection of bacterial mutants. J. Bacteriol. **63**: 399–406.

SERMONTI, G., and I. SPADA-SERMONTI, 1955 Genetic recombination in Streptomyces. Nature **176**: 121.

1956 Gene recombination in *Streptomyces coelicolor*. J. Gen. Microbiol. **15**: 609–616.

1959 Genetics of *Streptomyces coelicolor*. Annals. N.Y. Acad. Sci. **81**: 854–861.

SOMATIC VARIATION AFFECTING ERYTHROCYTE ANTIGENS

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THE red blood cells of man have been shown to be polymorphic with respect to the A agglutinogen in that most of the red cells are agglutinated by anti–A fluids and only a small fraction remain inagglutinable. It has been proposed that the inagglutinable cells are the progeny of bone marrow stem cells in which mutations have arisen (ATWOOD and SCHEINBERG 1958, 1959). Evidence for the mutational origin of the inagglutinable cells as opposed to the recombination hypothesis has been presented by ATWOOD (1958). He has shown that in an A_1B individual the predominant cell type is A_1B, i.e., capable of being agglutinated by anti–A_1, and anti–A_2 and anti–B fluids. A small fraction possesses only A_2B and a third fraction lacks both A antigenic factors, the latter frequency being about .001. Since the chromosomes in the predominant cell type are presumed to be in the repulsion phase, it is obvious that crossing over or a similar process leading only to coupling of A_2 and B has not occurred. Moreover the frequencies of the A_2B and B cells appear to be quite different, implying independent events.

It is well known that ionizing radiation increases the rate of germinal mutations, and presumably should also increase the rate of somatic mutations as well. If the inagglutinable cells arise as a result of mutation, it would be expected that there would be an increase in the frequency of these cells following irradiation. The experiments reported in this paper were carried out to test this hypothesis.

MATERIALS AND METHODS

SHAW and VERMUND (1959) reported that the red cells of pigeons, *Columbia livia*, react with *Phaseolus lunatus* seed extracts. STIMPFLING (1959) also observed that *P. lunatus* may be used for typing pigeon red cells. SHAW (1959) indicated further that the agglutinogen detected by *P. lunatus* seemingly is inherited as a simple dominant. SHAW and VERMUND (1959) reported that the pigeon is relatively radioresistant with the 30 day, 100 percent lethal dose being 2,500 rads. The pigeon was thus ideally suited for testing the effect of irradiation on the production of inagglutinable cells.

ATWOOD and SCHEINBERG (1958) described a procedure for the isolation of inagglutinable cells and for the determination of the inagglutinable cell frequency. With a few exceptions, the procedure described in that paper for the determination of the inagglutinable frequency was followed.

The volume of cells which were labelled with Cr-51 varied from 2 cc to 3 cc of packed cells, the cells being diluted with saline 1:4 prior to incubation. Prior to each run the labelled cells were centrifuged and the packed cells were diluted

with an equal volume of saline. These diluted cells averaged 10.3×10^6 counts per cc per minute. Agglutination of pigeon red cells with *P. lunatus* extracts proceeds very slowly at 4°C, the temperature at which the experiments with human red cells are carried out. Consequently, the phase of the experiment which involved agglutination was conducted at room temperature. In order to prevent bacterial growth during the run the agglutinin was treated with merthiolate 1:4000.

Separation of the agglutinated cells from the inagglutinables was accomplished by gravity separation in a burette. This process was initiated at room temperature, and after ten minutes of settling the supernatant fluid was drawn off and kept at 4°C until the next stage of agglutination. The agglutinated mass of cells was kept at 4°C for ten minutes for an additional period of settling, and the extruded supernatant fluid was added to the supernatant fluid from the initial separation. Samples for counting were diluted with saline to bring them into counting range, then were washed three times and the counts were made on the washed cells.

The corrections involved in the determination of the inagglutinable cell frequency have been discussed by ATWOOD and SCHEINBERG (1958). It has been shown that the anomalous rise following the achievement of equilibrium is due to the extrusion of inagglutinable cells from the agglutinated pellet. This phenomenon was also observed in the isotope dilution experiments with pigeon blood. The suggested correction (ATWOOD and SCHEINBERG 1958) does not fully correct for the rise observed in the experiments with pigeons. It was found empirically that if the correction for dilution involves both the addition of agglutinin and the red cells to be used as carrier cells and an additional correction is made only for sample loss. then the rise observed at the plateau region is reduced. Regardless of the procedure followed to obtain the inagglutinable cell frequency it does not alter the conclusions drawn from the observed data.

The pigeons were irradiated with a General Electric Maximar 100 at 100 kilovolts potential with five milliamps of current and a filter of one millimeter of aluminum. In the first experiment the bird was placed on its side and tied in position directly under the X-ray beam 30 cm from the source. Subsequent irradiation experiments were conducted with the bird in a lucite cylinder which was rotated under the X-ray beam to provide uniform total body exposure. A victoreen total dose "R" meter or rate meter was used to determine the dose received by the pigeon in air and was taken at a position which marked the central point of the bird. An estimate of the amount of shielding contributed by the pigeon was obtained in phantom runs using a dead pigeon in which the "R" meter probe was placed in the peritoneum and left during the irradiation. The 4π cobalt gamma source provided an exposure of 279r/minute to the peritoneum and an air dose of 335 roentgens.

The sequence of operations in the irradiation experiments was as follows: each bird was bled heavily, obtaining as much as 15 cc of blood; the inagglutinable cell frequency was determined by the isotope dilution method, and the bird was irradiated a day following bleeding. The extensive bleeding was carried out to

eliminate from the terminal red cell population many of the mature red cells prior to irradiation. RODMAN, EBAUGH and SPIVEY FOX (1957) indicate that the lifetime of pigeon red cells is 40 to 45 days. Therefore the postirradiation isotope dilution experiments were conducted at a time when most of the terminal red cells present at the time of irradiation would have been removed from the blood. The conditions present at the preirradiation experiment were closely adhered to in the postirradiation experiments. The titers of *Phaseolus lunatus* with the carrier cells in the initial experiment determined the titer used at each subsequent experiment. The pigeon providing the cells to be used as carrier in the initial experiment was also used in each subsequent isotope dilution experiment. The volume ratios of cells to agglutinin at the initial stage and the amount of carrier and agglutinin used at subsequent stages were the same for the pre- and postirradiation experiments.

RESULTS

The Phaseolus lunatus *agglutinin:* The Phaseolus extract which agglutinates red cells of pigeons is identical with or similar to the anti–A_2 antibody of man. This conclusion was based on the results of the following experiments. Human A_2 cells were used to absorb the Phaseolus agglutinin, and the agglutinin was then eluted from the cells by the method of WIENER (1957). The cells of six human A_1 bloods reacted with the eluate as did also the cells of eight pigeons. On the other hand, the cells of 11 pigeons tested at various times failed to react with the extract of *Dolichos biflorus*, a strong anti–A_1 reagent.

The inagglutinable cell frequency in pigeons: Isotope dilution experiments were carried out on homing pigeons using *Phaseolus lunatus* agglutinin. In Table 1 are listed the inagglutinable cell frequencies obtained at equilibrium. The level of inagglutinable cells found with pigeons is greater than that found for A_2 cells. The values reported by ATWOOD and SCHEINBERG (1958) for A_2 cells was .0109 and ranged from .0005 to .004 for A_1 cells. The variance of inagglutinable frequencies of pigeon blood is also comparable to that found with human blood if comparisons are made without regard to the blood type of the latter species. Part of the variance may be due to the presence of a mixed population of homozygotes and heterozygotes. The limited data indicate a cluster of frequencies about .1 and at least one frequency close to .01, the last frequency being obtained with blood

TABLE 1

Inagglutinable cell frequencies of the pigeon

Bird number	Frequency
921	.080
935	.026
954	.100
986	.0093*
2943	.046

* Equilibrium achieved at .0076 using human A cells as carrier.

presumably from a homozygote. The population from which the pigeons were drawn has been a closed flock since 1924. Linebreeding and even closer forms of inbreeding such as brother–sister mating and parent–offspring mating are practiced regularly. It would therefore, not be surprising to find a high frequency of homozygotes in this population. Unfortunately, a precise estimate of the inbreeding coefficient cannot be made since records of all matings are not kept.

The contributions to the variance of the inagglutinable frequency from causes other than homozygosity have been discussed (ATWOOD and SCHEINBERG 1958). An additional factor which might add to the variance particularly in pigeons is that due to polyspermy (HOLLANDER 1949). HOLLANDER (1959) informed the authors, however, that his observations and breeder's reports indicate that visible mosaics are found in less than one percent of birds from matings which could result in mosaics. It would appear that there would be no substantial contribution to the variance from polyspermy.

The variation observed in the inagglutinable frequencies between individuals is in marked contrast to the relative constancy in the inagglutinable frequency observed when retesting the same individual. ATWOOD (1959) has recently retested three human bloods a year after the first experiments were carried out and found their inagglutinable cell frequency unchanged.

The carrier effect: During the course of the initial irradiation experiments it was found desirable to obtain larger quantities of carrier cells than could be obtained from pigeon cells. Because both pigeon and human red cells possess the A_2 specificity, human A cells were chosen for this purpose. Following the attainment of equilibrium with pigeon red cells as carrier, human red cells were used. The addition of the human cells resulted in further removal of pigeon cells to the agglutinated pellet and a new equilibrium was reached lower than the first. The results of one such experiment are illustrated in Figure 1. The human A_1 blood and pigeon blood were chosen because they reacted to the same titer with *P. lunatus* extract. Human A_2 cells do not exhibit the carrier effect in isotope dilution experiments when human A_1 cells are used as carrier cells following the attainment of equilibrium with human A_2 cells. This is illustrated in Figure 2. Nor may a carrier effect be demonstrated in all pigeon cells. A detailed consideration of the carrier effect will be taken up in a subsequent publication.

In Table 2 are included the inagglutinable cell frequencies obtained using human A cells as carrier. In these experiments as in all the isotope dilution experiments completed to date each blood has been found to possess an inagglutinable cell fraction, there have been no exceptions. The levels of inagglutinable cells observed using human A cells as carrier are in general lower than those indicated in Table 1, but the variance of the frequencies suggests two classes of genotypes analogous to the suggested conclusion from the results given in Table 1.

Effect of irradiation on pigeons: The observations made by SHAW and VERMUND (1959) regarding the relative resistance of pigeons to ionizing irradiation have also been observed by the authors. All of the six pigeons given 1000r either with the G. E. Maximar 100 KVP (dose rate of 35–50r/minute) or the 4π cobalt source (dose rate of 335r/minute) are still alive four months following irradi-

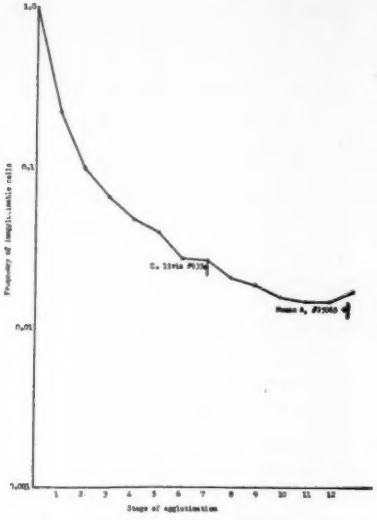

FIGURE 1.—The carrier effect.

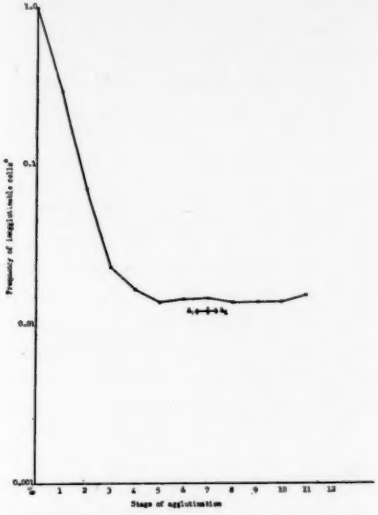

FIGURE 2.—Lack of carrier effect in human A blood.

TABLE 2

Isotope dilution frequencies in pigeons using human A cells as carrier

Bird number	Inagglutinable frequency
814	.0093
843	.0011
867	.0057
908	.00097
926	.0015
972	.0073*

* In this experiment human A carrier cells were added in the initial stage whereas in all other experiments the carrier was added beginning with the second stage.

ation; one bird given 1000r of X-rays more than nine months previously is also alive and all are active. Among the four females given 1000r of X-irradiation two have laid eggs, one of which was fertile but did not hatch. The intensity of the delivered dose may play a role since two birds given 1500r with 4π cobalt source were dead by 35 days, whereas SHAW and VERMUND report the 30 day 100 percent lethal dose as 2500 rads using X-rays.

The effect of ionizing radiations on the inagglutinable cell frequency in pigeons: The results of isotope dilution experiments on bloods taken before and after irradiation are given in Figures 3, 4 and 5. As can be seen from Figure 3, 51 days following X-ray exposure the frequency of inagglutinables had increased

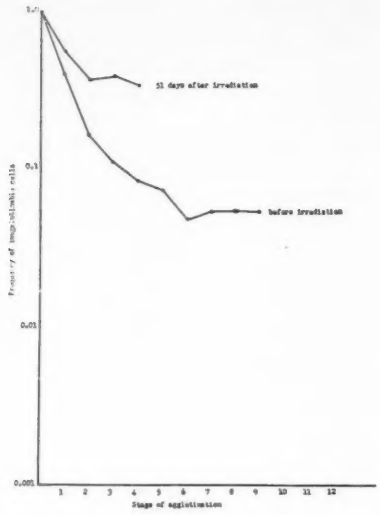

FIGURE 3.—Inagglutinable cell frequency in pigeon no. 2943.

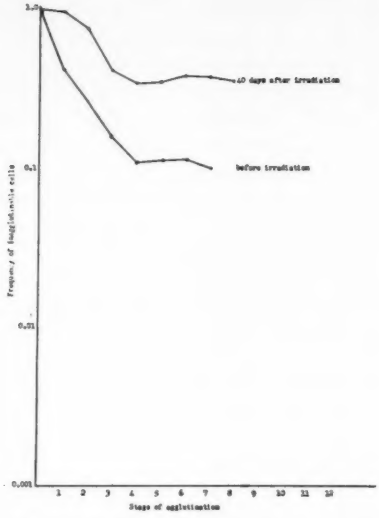

FIGURE 4.—Inagglutinable cell frequency in pigeon no. 954.

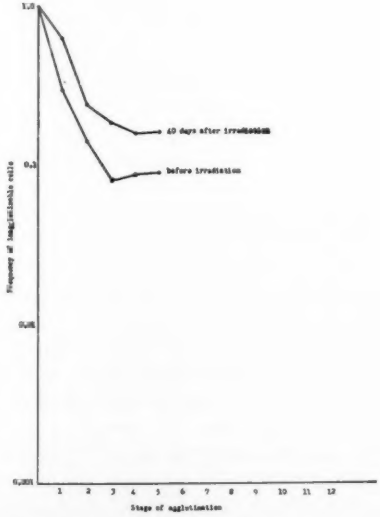

FIGURE 5.—Inagglutinable cell frequency in pigeon no. 921.

from .046 to .355. This particular bird was irradiated while lying on its side whereas all other birds exposed to X-rays were irradiated with the bird in a rotating lucite cylinder. The dose in air received by this bird was 1000r and the dose to the peritoneum was 630r. Pigeon No. 954 exhibited a threefold increase in inagglutinable cells at 40 days following irradiation with 700r in air (500r to the peritoneum). Pigeon No. 921 given an air dose of 1023r (peritoneal dose of 818r) showed an increase at 40 days postirradiation amounting only to 1½ times the preirradiation level, whereas it had been expected that increasing the dose would likewise increase the inagglutinable frequency. Other experiments which will be discussed indicate that at high doses there is a smaller increase in the number of inagglutinables detected which suggests a strong selection against those cells which would have been detected as inagglutinables. The first reaction upon retesting the blood of No. 921 was that an experimental error of some kind had been committed and resulted in the low value obtained. Therefore, this bird was bled seven days later and a fourfold increase in the frequency of inagglutinable cells was observed. The inagglutinable cell frequency was again determined 61 days later and was found essentially indistinguishable and in fact, lower than the preirradiation frequency, .051 to .081.

RODMAN, EBAUGH and SPIVEY FOX (1957) indicated that pigeons possess about 33 ml of red cells per kilogram of body weight. This would mean that in the procedure followed that from one third to one half of the blood volume was removed at each bleeding. Undoubtedly this stress resulted in marked hematopoiesis. It was therefore of great importance to determine whether these inagglutinable cells represented the aberrant progeny of radiation injured bone marrow cells or were merely newly formed cells. A number of experiments were carried out to test whether the inagglutinable cells were young cells.

Leucocytes when isolated from the terminal red cell population were found to be reactive with *P. lunatus* and conform to the red cell antigen type. When bone marrow cells are isolated, they too are found to be reactive with *P. lunatus*, and they too conform to the red cell antigen type. Consequently, young cells are reactive with *P. lunatus*. This observation is also confirmed by experiments of CALLENDER, POWELL, WITTS and YOUNG (1945) who tested the agglutinating properties of reticulocytes with anti–A sera and found that all but a small fraction of reticulocytes are agglutinable, analogous to the findings with terminal red cells. STIMPFLING (1959) informed the authors that the red cells of young newly hatched squabs are also reactive with *P. lunatus* extracts.

The inagglutinable cells were isolated from two pigeons using *P. lunatus* extract as the isolation medium. With the exception of *P. lunatus* the selected and unselected cells reacted identically with the seed extracts of *Alysicarpus vaginalis*, *Crotolaria grantiana*, and *Vicia villosa*, these three causing no agglutination and *Sesbania exalta* and *Coronilla varia* which agglutinated both selected and unselected cells. These data suggest that the *P. lunatus* negative cells are specifically nonreactive.

An isotope dilution experiment was carried out to determine the effect of bleeding alone. A bird was bled heavily and tested for its inagglutinable frequency

which was found to be .01. This bird was retested 33 days later and the observed frequency was .009, and duplicate determination made the following day gave a frequency of .0098. Bleeding by itself would therefore not account for the increased frequency of the inagglutinable cells following irradiation. It is concluded that the inagglutinable cells are not merely young cells.

Radiation effects on the inagglutinable frequency in man: The authors have been fortunate in obtaining samples of blood from a patient with polycythemia vera who had been treated with 2 mc of P^{32}. The experimental procedure followed to determine the inagglutinable frequency is that outlined by ATWOOD and SCHEINBERG (1958) with the exception of the method for computing the frequency as previously discussed. The data obtained are presented in Figure 6. The base inagglutinable frequency was obtained seven days following treatment at a time when no effect of the treatment would be expected. Blood had been obtained prior to treatment, but because of a sampling error its use is precluded. Figure 6 shows that as much as a tenfold increase in the frequency of inagglutinables was observed. This frequency has persisted almost unchanged for 173 days —considerably beyond the reputed lifetime of the red cell which is 120 days. The inagglutinable cells observed at this time are therefore presumably the progeny of bone marrow cells exposed to the P^{32} treatment. During and following the period of treatment, the hematocrit has remained constant and within the normal range.

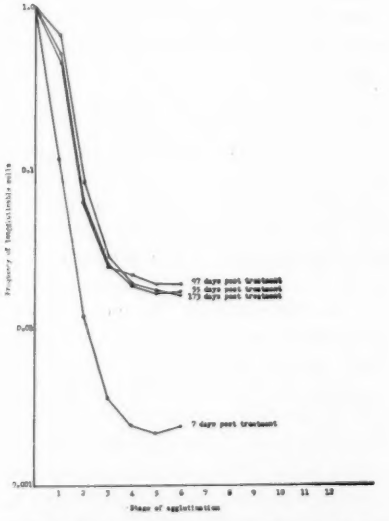

FIGURE 6.—Effect of P^{32} on the inagglutinable frequency in a woman with Polycythemia vera.

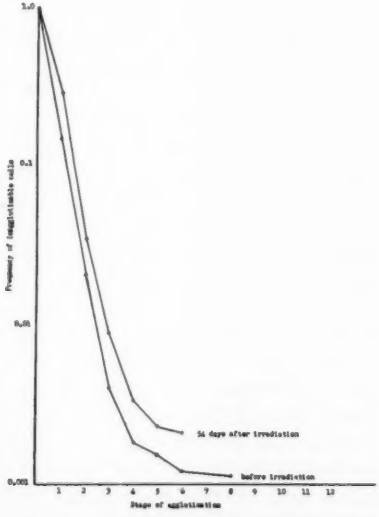

FIGURE 7.—Inagglutinable cell frequency in pigeon no. 893.

DR. ATWOOD of the University of Chicago has also carried out isotope dilution experiments on a patient with polycythemia vera who received 4 mc of P^{32}. ATWOOD has been kind enough to permit us to include the preliminary results he obtained with the blood of this patient. The inagglutinable frequency with anti–A_2 reagents prior to treatment was 2.1×10^{-3}; two weeks after treatment this value was now 10^{-2}, a fivefold increase in the frequency; by 30 days however this frequency had dropped to 3.9×10^{-3}, a little less than two times the increase over the initial value. The inagglutinable frequency on these same bloods using both anti–A_2 and anti–H was 6.5×10^{-5} prior to treatment and 1.7×10^{-4} at two weeks but had fallen to its preirradiation level 30 days after treatment. The comparison of ATWOOD's results with results obtained by the authors is reminiscent of the results observed with X-ray treatment of pigeons in that high doses did not result in a further increase in the frequency of inagglutinables but rather a decreased frequency suggesting increased selection against cells of the inagglutinable phenotype. Similar results have been obtained in other experiments with pigeons.

An experiment was carried out in which a pigeon was given 1040r of X-rays in air (832r intraperitoneally), and the isotope dilution experiments were conducted using human A_1 cells as carrier cells. The results presented in Figure 7 indicate a change in the inagglutinable frequency from .0011 to .0021. The dose rate in this experiment was 52 roentgens per minute. In order to obtain higher intensities of irradiation use has been made of the 4π cobalt source of the Naval Medical Institute, Bethesda. At the time of irradiation the air dose was 335 roentgens per minute, whereas the intraperitoneal dose was 279 roentgens per minute. Pigeon No. 908 received an air dose of 1224 roentgens (1004 roentgens to the peritoneum) and the inagglutinable frequency observed prior to irradiation was .001 and 49 days following irradiation had a level of .003. On the other hand, pigeon No. 926 received 610 roentgens in air (500 roentgens to the peritoneum) and demonstrated a marked increase in the inagglutinable frequency from .0014 to .018, 46 days following irradiation. These results are also reminiscent of the results observed with the P^{32} treatments of the polycythemics and similarly suggest a high level of selection against the inagglutinable cells in pigeons exposed to high doses of radiation. The possibility that heavy selection against the inagglutinable cells takes place with high doses of radiation implies gross nuclear and/or cytoplasmic damage as a result of the high levels of irradiation. Only those cells which suffer minor damage will survive and in this sense the extent of damage will be indicated by the longevity of the inagglutinable bone marrow stem cells.

A number of pigeons exposed to X-irradiation have been retested for their inagglutinable frequencies at various intervals following irradiation. These data are presented in Table 3.

It is evident that there is a decline in the frequency of the inagglutinables with time when comparisons are made with the first postirradiation experiment. This is attributed to the effect of selection, but there is still a higher level of inagglutinable cells than that observed prior to irradiation.

If the red cell lifetime of the pigeon is 40–45 days, and the total volume of cells

TABLE 3

Effect of irradiation on the frequency of inagglutinable cells

Pigeon no.	Type of irradiation received	Dose received		Pre-irradiation freq.	Inagglutinable frequency and number of days posttreatment							
		In air	Intraperitoneally		Days following irradiation	Freq.	Days following irradiation	Freq.	Days following irradiation	Freq.	Days	Freq.
2943	X-ray*	1000	630	.0460	51	.3550	126	.2520	221	.1700	...	....
954	X-ray*	700	500	.1080	40	.3370	109	.2150	204	.2000	432	.2411
986	X-ray*	945	745	.0076	..	....	104	.0360	208	.0128	...	....
893	X-ray†	1040	832	.0011	54	.0021	...	....	...	....	...	....
908	γ-ray†	1224	1004	.0010	49	.0030	...	....	...	....	...	....
926	γ-ray†	610	500	.0014	46	.018	120	.0042	192	.0050	...	....
951	γ-ray†	120	101	.0028	48	.0026	149	.0025	...	....	...	....

* Pigeon cells used as carrier.
† Human A_1 cells used as carrier.

is about 23×10^9 cells, and furthermore if a stem cell origin for the red cells is assumed, then there is a daily addition to the terminal population of about 5.4×10^8 cells. There is also a daily inagglutinable cell production in the case of No. 954 of 5.4×10^7 cells and in the irradiated population of at least 11.8×10^7 cells for over 200 days.

DISCUSSION

The data reported in this paper clearly indicate an effect of ionizing radiation on the frequency of "A" inagglutinable cells. It is not known whether the changes induced in the bone marrow cells affect primarily the chromosomes or involve a change in the cytoplasm. A cytoplasmic change would be expected not to persist, and therefore, long-term experiments are being continued to provide additional evidence regarding the nature of the change.

Somatic variability of red cells has been observed in patients where disease has apparently affected the agglutinability of these cells. GOLD *et al.* (1959) reported that red cells of a male patient with acute monoblastic leukemia were weakly agglutinated by anti–A grouping sera. Treatment with 6-mercaptopurine and prednisone increased the number of agglutinable cells from 2 to 35 percent, and when the treatment was discontinued, the level of the agglutinable cells fell to 20 percent. In this case there is evidence for the disruption of normal functions of bone marrow cells which could involve the genetic apparatus.

SUMMARY AND CONCLUSIONS

Isotope dilution experiments with the bloods of the pigeon confirm the existence of inagglutinable cells observed in man. The agglutinin used was *Phaseolus lunatus* seed extracts and the red cell agglutinogen found on the pigeon cells is

believed to be that of A_2. Human A cells when used as carrier result in a new lower equilibrium level than that achieved using only pigeon cells as carrier. The level of inagglutinable cells was increased in pigeons with either X-rays or gamma-rays from a cobalt source. Increases in the frequency of inagglutinable cells were also observed in human polycythemic patients treated with P^{32}. Doses around 500r have given a greater increase in the frequency of inagglutinable cells than that obtained with levels of 1000r.

Persistence of the increased levels of inagglutinable cells was observed in three pigeons retested at over 200 days following irradiation and in the polycythemic patient retested at 173 days posttreatment.

It is concluded that irradiation has resulted in damage to the bone marrow stem cells and provides additional evidence for the mutational origin of the antigen lacking cells.

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LITERATURE CITED

ATWOOD, K. C., 1958 The presence of A_2 erythrocytes in A_1 blood. Proc. Natl. Acad. Sci. U.S. **44**(10): 1054–1057.
1959 Personal communication.

ATWOOD, K. C., and S. L. SCHEINBERG, 1958 Somatic variation in human erythrocyte antigens. J. Cellular Comp. Physiol. **52** (Suppl. 1): 97–123.
1959 Isotope dilution method for assay of inagglutinable erythrocytes. Science **129**(3354): 963–964.

CALLENDER, S. T., E. O. POWELL, and L. J. WITTS, 1945 Life span of red cell in man. J. Pathol. Bacteriol. **57**: 129–139.

GOLD, E. R., GEOFFREY H. TOVEY, W. E. BENNEY, and F. J. W. LEWIS, 1959 Changes in the group A antigen in a case of leukaemia. Nature **183**(4665): 892–893.

HOLLANDER, WILLARD F., 1949 Bipaternity in pigeons. J. Heredity **40**(10): 271–277.
1959 Private communication.

RODMAN, GERALD P., FRANKLIN G. EBAUGH, JR., and M. R. SPIVEY FOX, 1957 The life span of the red blood cell and the red blood cell volume in the chicken, pigeon and duck as estimated by the use of $Na_2 Cr^{51} O_4$. Blood **12**(4): 355–366.

SHAW, DON, 1959 Private communication.

SHAW, DON, and HALVOR VERMUND, 1959 Modification of irradiation effects in the pigeon, *Columbia livia*. I. Effects of homologous and heterologous bone marrow transplants. Proc. Natl. Acad. Sci. U.S. **45**(1): 23–29.

STIMPFLING, JACK H., 1959 Private communication.

WIENER, W., 1957 Eluting red cell antibodies, a method and its application. Brit. J. Hematology **3**: 276–283.

GENETIC EFFECTS OF X-RAYS AND CATHODE RAYS ON OOCYTES OF HABROBRACON[1]

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DIFFERENCES in sensitivity of oocytes to X-irradiation depending upon stage in meiosis when treatment occurred, have been shown for a number of organisms including Habrobracon (WHITING 1938, 1941, 1945a, 1945b; ATWOOD, VON BORSTEL and WHITING 1956). Studies have also shown that death of Habrobracon embryos following irradiation of oocytes in late metaphase and in late prophase (diplotene) was attributable to chromosomal and not to cytoplasmic injury except for the highest doses applied to the late prophase oocytes (WHITING 1946, 1948, 1949, 1950, 1955). Differences in sensitivity of metaphase and prophase eggs have been shown to be great. WHITING's data indicate that about 400r is sufficient to kill roughly 50 percent of the embryos when oocytes have been treated in metaphase, but that about 12,000r is necessary if the X-rays are delivered to oocytes during late diplotene. The author's data have essentially confirmed these findings for metaphase oocytes (HEIDENTHAL, CLARK and GOWEN 1955). Both authors have demonstrated that X-rays induce both dominant and recessive lethals in metaphase oocytes (HEIDENTHAL 1952, 1953; WHITING 1954; ATWOOD, VON BORSTEL and WHITING 1956).

In addition to the comparisons of sensitivity with respect to stage, the author's interest has involved comparisons related to differences in *dose rate*. In a previous paper it has been shown that there is no difference in certain genetic effects produced by 124 kv and 50 Mev X-rays (HEIDENTHAL, CLARK and GOWEN 1955). It appears that the different methods by which the ionizations are produced are not important in this biological system. In these experiments the dose rates differed by a factor of about five.

In the present paper this work has been extended to include the effect of 800 kv (peak) electrons. In view of the results with 124 kv and 50 Mev similar effects might be expected (for equal dose and rate of accumulation of dose) if high energy cathode rays were used. However, on account of the higher dose rates associated with electron irradiation, differences in the genetic effect might be expected, provided the chemical reaction (or series of reactions) leading to the genetic change is sensitive to concentrations of radiation-produced active entities, e.g. free radicals.

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MATERIALS AND METHODS

Habrobracon is a small parasitic wasp which lays eggs on the caterpillar of a moth, *Ephestia kuehniella* Zeller. When the females are mated, about two thirds of the eggs are fertilized. These develop into females if the cross is made in such a way as to insure heterozygosity of the sex alleles. The unfertilized eggs develop into males. It has been shown repeatedly that if well-fed virgin females are incubated at 30°C and restrained from oviposition by removing the caterpillars from the vials for overnight, oocytes in first meiotic metaphase accumulate in the four eggs sacs at the distal end of the reproductive tract. If the females in this condition are irradiated and then placed with fresh caterpillars where they can lay eggs, only those eggs treated in first meiotic metaphase are laid, provided the egg-laying period is restricted to six hours. These eggs are examined for hatchability about 36 hours after the egg-laying period. If the aim is to study eggs treated in late prophase, the females which have already laid the metaphase eggs are again restrained from oviposition for overnight. The next day they are allowed to lay eggs for a two-hour period; then they are transferred to fresh caterpillars on which they are allowed to lay eggs for four hours. Eggs laid during the first of these two intervals are a mixture of those treated in late metaphase, diakinesis, and late prophase. Usually they are discarded. But the eggs laid during the four-hour period are, as a group, more uniform. These are the "prophase" eggs. These, too, can be studied for hatchability by examining them 36 hours after the four-hour egg-laying period. The above methods can be used for studies of eggs laid by virgin mothers and, if the surviving larvae are incubated, of adult male progeny. If the purpose is to study diploid eggs or female progeny, the mothers are mated immediately after they have been exposed as virgins to the radiation. Surviving diploid larvae can then be allowed to grow to eclosion as F_1 females. One can secure such virgin F_1 females either from irradiated metaphase or from prophase eggs, at will.

Methods as described above were used to study the hatchability of haploid eggs laid by virgin mothers of a special No. 33 stock which had been inbred shortly before the experiments started, by single brother-sister matings exclusively, for 18 generations. Studies were made of the hatchability of eggs irradiated in late metaphase, others in late prophase. A third series of experiments involved tests of F_1 virgin females for heterozygosity with respect to one or more recessive lethals. The females were reared from eggs irradiated during late prophase and later fertilized by untreated sperm of males from another vigorous wild type stock which had also been bred for 18 generations of brother-sister matings, stock no. 1. All virgin F_1 females were allowed to lay the haploid eggs separately in Stender dishes each of which was supplied with a stung caterpillar free from eggs. Transfers to fresh caterpillars in different dishes were made for each F_1 virgin on each of several successive days. Cultures were incubated at 30°C. Eggs laid by each female were counted each day. About 36 hours later, the cultures were examined for dead embryos and hatched eggs (larvae).

Use of the method just described depends upon the following considerations.

All eggs laid by F_1 virgins are haploid. This means that any eggs bearing one or more recessive lethals, effective during embryonic development, fail to hatch. Segregation involving one lethal should give a hatchability of about 50 percent. Independent assortment of two lethals should yield roughly 25 percent hatchability. The F_1 mothers have ten pairs of chromosomes. One might expect, then, that at sufficiently high doses, various combinations of independently assorted lethals, linked lethals, and semilethals would yield varying percentages of hatchability, depending upon the conditions.

In experiments in which metaphase eggs were studied, the low voltage X-rays were delivered by a conventional kx-4 roentgen ray machine. Conditions were kept as constant as possible; 124 kv; 10 ma; target-sample distance 33.9 cm; 102r per minute; no filter except that inherent in the tube. Later the machine was modified to give higher doses needed for the more resistant prophase work. Distance was shortened to 9.9 cm; amperage was increased to 20 ma; and voltage was increased to 157 kv. Under these conditions the machine delivered about 1200r per minute.

The source of the high energy electrons was a resonant transformer type electron beam generator (KNOWLTON, MAHN and RANFTL 1951; LAWTON, BELLAMY, HUNGATE, BRYANT and HALL 1951) that produced electrons of 800 kv (peak) energy. Dose was accumulated at a rate of about 200 to 5000 roentgens per *second*. Irradiation dose was measured by using a specially constructed air ionization chamber. Dose is expressed in roentgens, where one r represents an energy absorption of 5.34×10^{12} ev/gm of air.

During the irradiation the wasps were held in a thin walled, flat cylinder type of enclosure, which was made from 0.003 inch thick sheet polyethylene and was 2½ inches in diameter and ⅛ inch in height. The reason for restricting the height of the enclosure was to insure that the wasps would always be in the same part of the electron beam pattern. As many as 75 wasps were irradiated at a time. The absorbing thickness, i.e., wasp plus polyethylene container wall, was estimated to be less than 1 mm (0.1 gr/cm^2), which is well within the total penetration range of the electron (0.3 gr/cm^2) and in the range where the dose would be nearly uniform throughout the thickness.

RESULTS AND DISCUSSION

The data on hatchability of eggs irradiated in first meiotic metaphase and in prophase are given in Table 1. At a dosage of 1000r, what appears to be a significant difference in viability of embryos is apparent; 13.6 percent for X-rays and 20.0 percent for cathode rays. Note, however, that at 1500r no such significant difference appears. Below 1000r, the doses are less certain. Studies of doses above 1500r are not feasible because the hatchability percentages are so low. The data for prophase eggs treated with 12,000r show no difference in hatchability: 40.5 percent for X-rays and 41.1 percent for cathode rays. At a dose of 28,000r, the data show hatchability percentages of 5.5 (author's data) and 9.5 (WHITING 1945) for X-rays; 9.7 for cathode rays. If within these three values, comparisons

TABLE 1

Hatchability of first meiotic prophase and metaphase eggs treated with low voltage X-rays and with cathode rays

Dose	Stage treated	Kind of radiation	Number of experiments	Number of eggs	Number of larvae	Percent eggs hatched
Control			3	1,735	1,606	92.6 ± 0.6
1,000r	First meiotic metaphase	X-rays	4	952	129	13.6 ± 1.2
		cathode	9	1,598	320	20.0 ± 1.2
1,500r	First meiotic metaphase	X-rays	15	2,648	156	5.9 ± 0.5
		cathode	5	881	37	4.2 ± 0.7
Control			2	355	328	92.4 ± 0.6
12,000r	First meiotic prophase	X-rays	3	629	255	40.5 ± 2.0
		cathode	3	479	197	41.1 ± 2.5
28,000r	First meiotic prophase	X-rays	3	495	27	5.5 ± 1.1
		X-rays*	3	772	73	9.5 ± 1.1
		cathode	8	1,629	157	9.7 ± 0.7

* Compiled from data of WHITING 1945.

are made involving dose rate as between X-rays and cathode rays, a small discrepancy is shown (5.5 and 9.7 compared to 9.5 and 9.7). If the first of the two values is regarded as representing a significant difference, then a small difference in hatchability has to be related to a large difference in dose rate since for the prophase work the cathode rate exceeded the X-ray rate by a factor as large as 250 × (5000r per second for cathode rays and 20r per second for X-rays). Furthermore, there is obviously no difference between X-ray data of WHITING and the cathode ray data. It should also be noted that the data for prophase eggs treated with 12,000r rather than 28,000r, show no significant difference (40.5 percent for X-rays and 41.1 percent for cathode rays).

Data on the incidence of recessive lethal heterozygotes among all F_1 females are summarized in the histograms of Figure 1. It will be recalled that the F_1 virgins which were tested by counts of F_2 eggs, developed from eggs irradiated by electrons, in the late prophase stage. The dose was 12,000r. The percentage of females carrying at least one recessive lethal is 7.4 (14/189). Somewhat comparable data, for X-rays, is found in the work of others (ATWOOD, VON BORSTEL and WHITING 1956). Table 1 of this paper shows that for one experiment, with dosage of 15,000r, the percentage of F_1 females carrying recessive lethals is 10.5 (14 heterozygotes/134 females tested); for a second experiment at the same dose the percentage is 14.4 (17/118). These two experiments taken together give 12.3 percent (31/252). The difference between 7.4 percent for cathode rays when dose is 12,000r and 12.3 percent for X-rays when dose is 15,000r, suggests that if difference in dose rate delivered by the two sources of radiation produces a difference in effect, this difference would have to be very small. Whichever type of radiation would be used, a difference of 3000r in dose would be expected to yield a corresponding difference in hatchability. Since the percentages yielded by 12,000r and 15,000r are relatively low, the difference due to ± 3000r would be

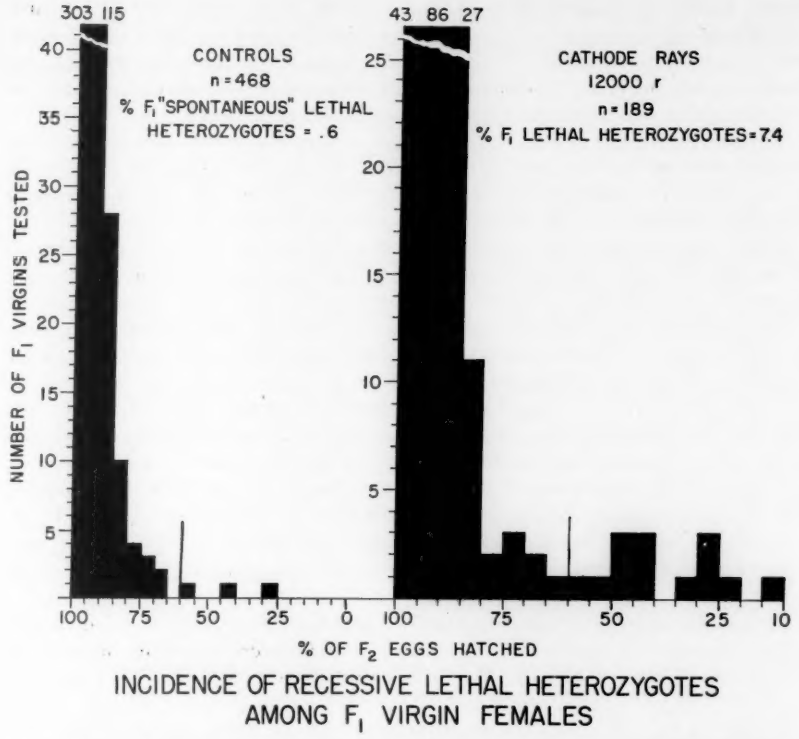
303 115
CONTROLS
n = 468
% F1 "SPONTANEOUS" LETHAL HETEROZYGOTES = .6
43 86 27
CATHODE RAYS
12000 r
n = 189
% F1 LETHAL HETEROZYGOTES = 7.4
NUMBER OF F1 VIRGINS TESTED
% OF F2 EGGS HATCHED
INCIDENCE OF RECESSIVE LETHAL HETEROZYGOTES AMONG F1 VIRGIN FEMALES

FIGURE 1.—Incidence of recessive lethal heterozygotes among F_1 females which developed from oocytes treated with 12,000 roentgens of cathode radiation during late, first meiotic prophase.

expected to be only a few percent. If one takes errors of variability into consideration, a difference of 4.9 percent seems to be reasonable.

Reference to Figure 1 will show that among controls, there were three females which tested as though they were heterozygous for one or more recessive lethals. These are thought of as "spontaneous" lethals. Such a rate can be regarded as a kind of composite for both eggs and sperm from which the F_1 mothers developed, since in a given case a lethal could have occurred in either an egg or a sperm. Conceivably the mother of an F_1 could transmit a lethal which she had inherited from her mother since females are diploid. A mother would not have received a lethal from her father except as it would have originated in his germ cells, because males are haploid and cannot exist as bearers of lethals. It is probable then that most "spontaneous" lethals should be thought of as having been transmitted to F_1 daughters from their mothers, although spontaneous rate in the gametes of the fathers cannot be neglected. It should also be remembered that stocks had been

closely inbred by 18 generations of single brother-sister mating and that during this process efforts were made to select for viability. Furthermore, if one deals with a natural host-parasite relationship, one cannot invariably be sure that eggs die because of the genetic constitution. This means that only very occasionally a count might be somewhat reduced by such environmental conditions.

Further inspection of Figure 1 will show that of the 189 F_1 females tested, a total of 14 were heterozygous for one or more lethals, that of these 14, eight can be regarded as heterozygous for one lethal, five for two lethals, and one for three or more. However, it is possible that this assumption is too simple to explain the realities fully. On the basis of a Poisson distribution, the incidence of heterozygotes with two and with three lethals is too high. A decisive reason for this discrepancy is not readily available. However, it seems possible that recombination and assortment of sublethals might occasionally modify the hatchability counts in such a way as to make it *seem* as though the counts were reflecting the simpler condition of a single or double recessive. A comparison of control and experimental data as given in Figure 1 is of some interest in this connection. Here one can see that there were fewer F_1 females among experimentals than among controls, which showed a hatchability of F_2 eggs as great, or greater than 90 percent. Among controls there were 89 percent of the females which laid F_2 eggs yielding this high hatchability. This value compares with only 68 percent among F_1 experimental females. Such observations suggest confirmation of a widely held conclusion, that ionizing radiation produces, apart from striking mutations, many other genetic changes which are slightly deleterious in their effects. Conceivably such sublethals may have had some effect, possibly not very great, on the hatchability counts of F_2 eggs laid by F_1 mothers listed as heterozygotes for one or more lethals. At the present time this is a technically difficult matter to settle decisively.

SUMMARY

This paper is concerned with the problem of difference in *dose rate*. Are genetic effects the same, or different, when dose rates are widely different, but total dose accumulated the same? A conventional 124 kv X-ray machine was used for the low dose rate work; an electron beam generator which yielded a rate as high as 5000r per *second* was the source of high dose rates. The materials irradiated were Habrobracon oocytes in first meiotic prophase and metaphase stages. These were studied for hatchability (presence of dominant and recessive lethals). F_1 virgin females were reared from prophase oocytes treated with 12,000r. These were tested for heterozygosity with respect to recessive lethals. The data so accumulated indicate that with these materials, when total doses of X-rays and cathode rays are the same, the wide difference in dose rate has no significant effect.

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LITERATURE CITED

ATWOOD, K. C., R. C. VON BORSTEL, and A. R. WHITING, 1956 An influence of ploidy on the time of expression of dominant lethal mutations in Habrobracon. Genetics **41**: 804–815.

HEIDENTHAL, G., 1952 X-ray induced recessive lethals in Habrobracon. (Abstr.) Genetics **37**: 590.

1953 A comparison of X-ray induced dominant and recessive lethals in first meiotic metaphase eggs and in sperm of Habrobracon. (Abstr.) Genetics **38**: 668.

HEIDENTHAL, G., L. B. CLARK, and J. W. GOWEN, 1955 Comparative effectiveness of roentgen rays of 124 kv and 50 Mev on Habrobracon eggs treated in first meiotic metaphase. Am. J. Roentgenol. Radium Therapy **74**: 677–685.

KNOWLTON, J. A., G. R. MAHN, and J. W. RANFTL, 1951 The resonant transformer: a source of high energy electrons. Electronics **12**: 64–66.

LAWTON, E. J., W. D. BELLAMY, R. E. HUNGATE, M. P. BRYANT, and E. HALL, 1951 Studies on changes produced in wood exposed to high velocity electrons. Tappi **34**: 113A.

WHITING, A. R., 1938 Sensitivity to X-rays of stages in oogenesis of Habrobracon. Records Genet. Soc. Am. **7**: 89.

1941 X-ray sensitivity of first meiotic prophase and metaphase in Habrobracon eggs. Records Genet. Soc. Am. **10**: 174.

1945a Dominant lethality and correlated chromosomal effects in Habrobracon eggs X-rayed in diplotene and in late metaphase I. Biol. Bull. **89**: 61–71.

1945b Effects of X-rays on hatchability and on chromosomes of Habrobracon eggs treated in first meiotic prophase and metaphase. Am. Naturalist **79**: 193–227.

1946 Motherless males from irradiated eggs. Science **103**: 219–220.

1948 Incidence and origin of androgenetic males in X-rayed Habrobracon eggs. Biol. Bull. **95**: 354–360.

1949 Androgenesis, a differentiator of cytoplasmic injury induced by X-rays in Habrobracon eggs. Biol. Bull. **97**: 210–220.

1950 Absence of mutagenic action of X-rayed cytoplasm in Habrobracon. Proc. Natl. Acad. Sci. U.S. **36**: 368–372.

1954 The effect of oxygen on the frequency of X-ray induced mutations on Habrobracon eggs. Genetics **39**: 851–858.

1955 Androgenesis as evidence for the nature of X-ray induced injury. Radiation Res. **2**: 71–78.

THE STRUCTURE OF THE A MATING TYPE LOCUS IN COPRINUS LAGOPUS

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IN basidiomycetes with a tetrapolar mating system, compatibility between haploid monocaryons is controlled by the action of alleles at two independent loci *A* and *B*. If two monocaryons possess different alleles at both loci they may, by exchanging nuclei, form stable binucleate dicaryons. If they possess common alleles at either or both loci, they are incompatible but may form a heterocaryon. In many species the dicaryon is easily recognized by the presence of clamp connexions between adjacent cells. In the Hymenomycetes each mating type locus has a large number of alleles which has been calculated to be in the order of magnitude of 100 per locus in natural populations (WHITEHOUSE 1949). Ninety-six *A* alleles and 56 *B* alleles were found in a recent survey of *Schizophyllum commune* (RAPER, KRONGELB, and BAXTER 1958a). KNIEP (1930) was the first to find that nonparental alleles may occur at both loci in single fruit bodies of *S. commune*. Two such nonparental *A* factors, when intercrossed, gave rise to a small proportion of the original *A* factors (six in 33 or 18.2 percent). PAPAZIAN (1951) also found nonparental *A* alleles in tetrads and random spores of *S. commune* and attributed their origin to crossing over between subunits of the *A* locus. KNIEP's earlier data can also be explained in this way. RAPER, BAXTER, and MIDDLETON (1958b) have shown that both loci of *S. commune* are each made up of at least two subunits. The frequency of recombination between subunits at the *A* locus varied between 0.9 and 15.9 percent according to which stocks were intercrossed. Recombinant mating type factors have also been described in the tetrapolar species *Collybia velutipes* (TAKEMARU 1957) and *Pleurotus ostreatus* (TERAKAWA 1957; RAPER *et al.* 1958b).

This paper reports studies of recombination within the *A* mating type locus of *Coprinus lagopus* using linked markers. A preliminary report has already appeared (DAY 1959b).

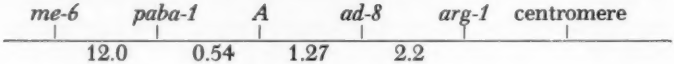
MATERIALS AND METHODS

Wild type stocks H1 (A_5B_5) and H9 (A_6B_6) were isolated from a single fruit body growing on a manure heap at Bayford in June 1956. Mutants 2242 (*ad-8*), 2246 (*arg-1*), 2214 (*me-6*), 2519 (*paba-1*), with requirements for adenine, arginine, methionine and para-aminobenzoic acid respectively, were induced in stock H9 by ANDERSON (1959). Recombinant stocks were obtained from crosses with H1. In addition tester stocks of the following mating types—A_5B_1, A_6B_1, A_2B_5 and A_2B_6—were obtainted by crossing H1 and H9 with stock 68 (A_2B_1). The

origin of stock 68 and the culture media used were given by DAY (1959a). Basidiospores were germinated on dung decoction (100 gm horse dung boiled in one L water) cleared by filtration through diatomaceous silica and solidified with two percent agar. Tetrads were isolated with a glass microneedle held in a Singer micromanipulator. The spores were separated and transferred to dung agar by hand using a glass needle with a small bead at the tip.

Mating types were determined by pairing unknown and tester stocks on complete medium in Petri dishes, 16 matings per dish. Clamp formation was scored after three days' incubation at 28°C.

The approximate linkage values and order of the *A* linked markers used are shown below (DAY and ANDERSON, unpublished).



RESULTS

Analysis of random spores: Several crosses were made between stocks bearing markers linked with the *A* locus before the order of the markers had been established. Cultures produced from basidiospores from these crosses were tested for *A* factors compatible with both parental *A* factors, A_5 and A_6, in matings with A_5B_1 and A_6B_1 tester stocks. From a total of 1213 cultures tested, two (A_xB_5 and $A_{x'}B_5$) with stable nonparental *A* reactions were found. Their genotypes and the crosses from which they came are given in Table 1.

The A_xB_5 culture was crossed with an A_6B_6 stock and the dicaryon was fruited. The alleles A_x and A_6 segregated normally among 130 random basidiospore cultures tested for mating type. A recombinant culture, A_xB_6, was selected and mated with $A_{x'}B_5$. The mating was incompatible, and it was concluded that A_x and $A_{x'}$ are identical. The genotypes of the two cultures with nonparental *A* reactions are consistent with their each having arisen as the result of a single cross-

TABLE 1

Crosses which gave nonparental A *alleles*

Cross	Nonparental *A* stock	Progeny tested
me-6$^+$ A_6 *arg-1* B_6		36 prototrophs
×	*me-6*$^+$ A_x *arg-1*$^+$ B_5	
me-6 A_5 *arg-1*$^+$ B_5		100 auxotrophs
A_6 *ad-8* *arg-1*$^+$ B_5		83 prototrophs
×	$A_{x'}$ *ad-8*$^+$ *arg-1* B_5	
A_5 *ad-8*$^+$ *arg-1* B_6		613 auxotrophs

over between subunits of the *A* locus, but the reciprocal recombinants were not recovered.

Analysis of tetrads: In a study of the effects of temperature and marker combinations on patterns of crossing over within a region of the *A* chromosome (DAY and SWIEZYNSKI, unpublished) some 688 tetrads were available from three crosses and these were examined for nonparental *A* and *B* factors. Four tetrads with nonparental *A* factors were found, but in one of the tetrads only three spores germinated. The constitution of the tetrads and the crosses from which they were produced are given in Table 2. No cultures with nonparental *B* reactions and no tetrads showing 3:1 segregations of the *A* or *B* loci were found.

Two cultures with nonparental *A* reactions were found in each of the tetrads 2397–2400 and 2689–2692. These cultures were mated with each other and with cultures carrying the A_x factor already recovered. The results of these matings are given in Table 3.

The test matings show that two new *A* specificities were present in each tetrad, one of which was identical with that of A_x. The other was different from A_x and was designated A_y. A recombinant stock, A_yB_1, was produced by crossing culture 2398 (A_yB_5) with stock 68(A_xB_1). The results of crosses with the A_yB_1 stock are also shown in Table 3.

TABLE 2

Tetrads with nonparental A *reactions recovered from crosses*

(1.) *me-6* A_6 *ad-8 arg-1*$^+$ B_5 × *me-6*$^+$ A_5 *ad-8*$^+$ *arg-1* B_6 (203 tetrads)

(2.) *me-6*$^+$ A_5 *ad-8 arg-1*$^+$ B_6 × *me-6* A_6 *ad-8*$^+$ *arg-1* B_5 (220 tetrads)

Cross	Spore no.	Genotype	A_xB_1	A_yB_1†
(1.)	2397	*me-6*$^+$ A_5 *ad-8*$^+$ *arg-1* B_5	−	+
	2398	*me-6*$^+$ A_y *ad-8 arg-1*$^+$ B_5	+	+
	2399	*me-6* A_x *ad-8*$^+$ *arg-1* B_6	+	+
	2400	*me-6* A_6 *ad-8 arg-1*$^+$ B_6	+	−
(1.)	2413	* *me-6*$^+$ A? *ad-8*$^+$ *arg-1*$^+$ B_6	+	+
	2414	*me-6* A_6 *ad-8 arg-1*$^+$ B_5	+	−
	2415	*me-6* $^+$ A_5 *ad-8*$^+$ *arg-1* B_5	−	+
	2416	*me-6* A_6 *ad-8 arg-1*$^+$ B_6	+	−
(2.)	2689	*me-6* A_x *ad-8 arg-1*$^+$ B_6	+	+
	2690	*me-6*$^+$ A_y *ad-8*$^+$ *arg-1* B_5	+	+
	2691	*me-6*$^+$ A_5 *ad-8 arg-1*$^+$ B_6	−	+
	2692	*me-6* A_6 *ad-8*$^+$ *arg-1* B_5	+	−
(2.)	1957	* *me-6*$^+$ A? *ad-8*$^+$ *arg-1*$^+$ B_5	+	+
	1958	*me-6*$^+$ A_5 *ad-8 arg-1*$^+$ B_6	−	+
	1959	*me-6* A_6 *ad-8*$^+$ *arg-1* B_6	+	−
	1960	*(me-6 A? ad-8 arg-1* B_5)		

* Phenotypes, genotypes discussed in text.
† + = compatible; − = incompatible.

TABLE 3

Results of intercrossing cultures with nonparental A *factors*

		2398 A_yB_5	2399 A_xB_6	2689 A_xB_6	2690 A_yB_5	A_xB_5	A_xB_6	A_yB_1
2398	A_yB_5	..	+	+	(−)*	(−)	+	−
2399	A_xB_6	..	..	(−)	+	−	(−)	+
2689	A_xB_6	..	..	..	+	−	(−)	+
2690	A_yB_5	..	..	..	..	(−)	+	−
	A_xB_5	..	..	..	..	..	−	+
	A_xB_6	..	..	..	..	..		+
2413	$A?B_6$	..	.	..	..	+	(−)	+
1957	$A?B_5$	..	..	..	..	(−)	+	+

* (−) = Incompatibility due wholly or in part to common *B* factors.

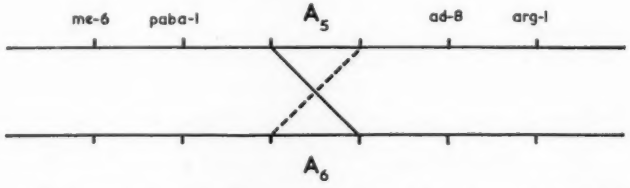


FIGURE 1.—Position of crossover recombining subunits of the *A* locus. A_x recombinant shown by broken line. A_y recombinant shown by unbroken line.

Both tetrads show the recombination of adjacent markers that would be expected if the only crossover within the region *me-6–arg-1* was between subunits of the *A* locus. Two recombinants (2398 and 2690) reacted as A_y, whereas the reciprocal recombinants (2399 and 2689) reacted as A_x (in Figure 1, the unbroken line and the broken line, respectively).

Tetrad 2413–16 gave one culture (2413) with a nonparental *A* reaction and also showed a 3:1 segregation for arginine independence. Culture 2413 was compatible with A_5B_1, A_6B_1, A_xB_5 and A_yB_1. The dicaryons produced by crossing 2413 with A_5B_1 and A_6B_1 were fruited, and basidiospore cultures from each cross were tested. The markers *me-6*, A_6 and *ad-8* segregated in the cross with A_5B_1 and markers A_5 and *arg-1* segregated in the cross with A_6B_1. The original culture showed occasional clamp connexions which failed to fuse with their neighboring cells (false clamps). Such clamps are produced by common *B* heterocaryons (SWIEZYNSKI and DAY 1960). It seems likely that the culture was originally disomic with two *A* chromosomes, *me-6* A_6 *ad-8 arg-1*$^+$/*me-6*$^+$ A_5 *ad-8*$^+$ *arg-1* and subsequently broke down to form a common *B* heterocaryon. The component

nuclei of the heterocaryon were sampled in the testcrosses which were fruited. PITTENGER (1954) has reported similar examples of disomics forming heterocaryons in *Neurospora crassa*.

Culture 1957 from the incomplete tetrad bore false clamps and was also compatible with the tester stocks A_5B_1, A_6B_1, A_zB_6 and A_yB_1. This culture produced oidia, which are uninucleate, and these, when sown, yielded 11 subcultures which were all *me-6*$^+$ A_5 *ad-8 arg-1*$^+$ B_5. It is possible that the missing culture, (spore 1960) was *me-6* A_6 *ad-8*$^+$ *arg-1* B_5 and was accidentally placed on the same slant as spore 1957 during the transfer of the tetrad to dung agar.

Analysis of prototrophs selected from crosses of linked auxotrophs in repulsion: The data from the tetrads established that the factors A_5 and A_6 were composed of two subunits each and that crossing over between them gave rise to the expected reciprocal products. An attempt was then made to recover more than two crossover products from crosses of $A_5 \times A_6$. There is no direct means of selecting nonparental *A* factors. This difficulty was overcome by crossing stocks with two linked auxotrophic markers, one on either side of the *A* locus, in repulsion. If crossing over occurred between or within the subunits of the *A* locus, this would, provided it was the only crossover within the interval bounded by the auxotrophic markers, bring about their recombination and give rise to a prototrophic recombinant and the reciprocal double mutant. Hence, half of the products of all single crossovers within the *A* locus would be recovered among the prototrophs. Since the distance of each marker from the *A* locus is known, the relative frequencies of prototrophs with parental and nonparental *A* reactions would give some indication of the distance between the subunits. If crossing over within the subunits occurred, more than two specificities would be found among the nonparental *A* factors.

The markers *paba-1* and *ad-8*, respectively 0.54 and 1.27 crossover units from *A*, were intercrossed in both repulsion crosses. Prototrophs were selected from random basidiospores spread on minimal medium and were mated with A_5B_1 and A_6B_1 to select cultures with nonparental *A* factors. The nonparental *A* cultures were either monocaryons or had clamp connexions. Both types were again mated with A_5B_1 and A_6B_1, and with A_zB_1 and A_yB_1. A summary of the results is shown in Table 4.

The monocaryon stocks with nonparental *A* reactions from each cross were only of one kind, A_x or A_y. This shows that either crossing over within subunits did not take place sufficiently frequently for the products to be recovered in this experiment or that the crossover products were not detected by the tests employed. Estimates of the frequency of crossing over between the markers, obtained by doubling the frequency of prototrophs, were 1.85 percent for the first cross and 1.04 percent for the second cross. The expected frequency was $0.54 + 1.27 = 1.81$ percent. The lower frequency (1.04 percent) in the second cross could have been due to differences in genetic background between the two crosses. This is supported by the lower frequency of nonparental *A* factors recovered, namely 3.61 percent in the second cross against 4.11 percent in the first cross. However, if we

TABLE 4

Summary of prototrophs isolated with the aid of linked selective markers

	(1) *paba-1* A_s *ad-8*$^+$ B_5 × *paba-1*$^+$ A_g *ad-8* B_6	(2) *paba-1*$^+$ A_s *ad-8* B_5 × *paba-1* A_6 *ad-8*$^+$ B_6	(3) *paba-1* A_x *ad-8*$^+$ B_5 × *paba-1*$^+$ A_y *ad-8* B_1
Percent prototrophs (×2)	1.85	1.04	1.18
Monocaryons			
A_s	246	655	30
A_g	739	267	0
A_x	43	0	208
A_y	0	36	380
Parental *A* ratios	1:3.00	1:2.45	1:1.81
Stocks with clamps	19	38	24
Total	1047	996	642

accept the interval between *paba-1* and *ad-8* as 1.81 units, then we may calculate the distance between the subunits of the *A* locus from each cross as

$$\frac{43}{1028} \times 1.81 = 0.076 \text{ and } \frac{36}{958} \times 1.81 = 0.068$$

(average = 0.072 units).

The relative frequency of crossing over expected in the two intervals, one on each side of the *A* locus, was 0.54:1.27 or 1:2.35. As shown in Table 4 the frequencies obtained were 1:3 and 1:2.45 in the two crosses.

All 19 stocks with clamps from the first cross in Table 4 were subcultured to sterile dung to test their ability to fruit. All except one fruited readily. A_s and A_g segregated in the progeny of four of these fruited cultures selected at random, no other factors being found in the samples (32 colonies) tested. Adenine and PABA requirements segregated in two progenies while only one requirement segregated in the other two progenies. These results show that the four dicaryons which were progeny tested most likely arose by crossing between complementary compatible strains in the original platings of basidiospores to minimal medium.

Other stocks bearing clamps, produced in the second cross, were tested with similar results. Of 11 stocks tested six failed to fruit. These latter stocks produced many false clamps and appeared to be common *B* heterocaryons.

Reconstitution of parental A *factors:* An additional proof that crossing over was the mechanism by which the nonparental *A* factors arose was obtained by crossing the two stocks *paba-1* A_x *ad-8*$^+$ B_5 × *paba-1*$^+$ A_y *ad-8* B_1. The results are summarized in the last column of Table 4. Of the 618 monocaryon prototrophs tested 30 had nonparental *A* factors and were all incompatible with A_s. Thus, one of the original parental *A* factors was recovered by crossing the recombinant factors A_x and A_y. The distance between the subunits calculated from this cross was 0.088 units and is in fair agreement with the results presented above.

DISCUSSION

The structure of genes concerned with incompatibility has now been investigated in several different organisms. In some self incompatible flowering plants the *S* gene controlling incompatibility has been shown to have a two subunit structure in which one subunit controls stylar specificity and the other pollen specificity. LEWIS and CROWE (1953) working with *Prunus avium* and PANDEY (1956) working with *Trifolium pratense* and *T. repens* have shown that mutations to self-fertility (S.F.) may be induced at either subunit by X-ray treatment. S.F. mutations of the pollen subunit are easier to screen than S.F. mutations of the style subunit. BREWBAKER and SHAPIRO (1958) found that nonparental alleles may arise in *Petunia inflata* as a result of crossing over between the subunits so that an *S* allele is produced which has, say, S_1 pollen specificity but S_2 style specificity and is consequently self-fertile.

LEUPOLD (1958) has shown that the mating type gene of the yeast *Schizosaccharomyces pombe* has two subunits which are thought to have arisen by duplication of a locus with two alleles, + and −, controlling heterothallism. One subunit, however, only has one functional allele +, the other allele 0, is nonfunctional. The genotypes associated with the '+' phenotype are ++ or +0, while the genotype for the '−' phenotype is −0 and that for the homothallic phenotype is −+. Thus when a homothallic recombinant (−+) is produced by crossing ++ × −0 a reciprocal '+' strain is formed, +0 which can be distinguished from ++ by genetic tests. The homothallic recombinant is analogous to the self-fertile recombinant of Petunia.

RAPER *et al.* (1958b) and DAY and HOLLIDAY (1959) have discussed the relationship between the subunits of the *A* and *B* loci of *Schizophyllum commune*. Here the subunits of each locus appear to be functionally indistinguishable and if a monocaryon has different alleles at both pairs of subunits (A_{1-2}, B_{1-2}) it is not self-fertile. If both subunits do have the same function, the simplest hypothesis is that a mating is compatible if at least three of the four subunits of each locus, brought together in a common cytoplasm, are different. These considerations also apply to the *A* locus of Coprinus (e.g., A_{1-2} is incompatible with A_{1-2}, A_{2-1}, A_{1-1} and A_{2-2} but is compatible with A_{1-4}, A_{4-1}, A_{3-4} and A_{3-3}).

If the subunits of the *A* locus of Coprinus or Schizophyllum have evolved by duplication of a pre-existing unit then it would be reasonable to expect that both subunits of a single locus might sometimes be identical. The reactions of three such loci (A_{1-1}, A_{2-2} and A_{3-3}) are shown above. With n different subunit specificities, which can be imagined as an allelic series, there are n^2 possible pairs, n of which will be identical pairs, $n^2 - n$ will be nonidentical pairs. With n^2 *A* factors the total number of possible different matings is n^4, and if we accept the restriction that at least three different subunits must be present for a mating between any two *A* factors to be fertile, the number of fertile matings can be calculated as follows:

(1) n identical pairs are each compatible with $(n-1)(n-2)$ nonidentical pairs

(2) $n(n-1)$ nonidentical pairs are each compatible with n^2-4 pairs.

The total fraction of compatible matings $=$

$$\frac{n(n-1)(n-2)+n(n-1)(n-2)(n+2)}{n^4}=\frac{n(n-1)(n-2)(n+3)}{n^4}=\frac{n^3-7n+6}{n^3}$$

Curve (a) in Figure 2 shows the percentage of fertile matings plotted against values of n between three and 30.

It can be seen that even for low values of n, between 10 and 20, the maximal frequency of compatible matings is already high, 93.6 to 98.3 percent.

In a collection of 114 strains of *S. commune* 97.05 percent of random matings between *A* factors were fertile (RAPER *et al.* 1958a), a condition which could be satisfied by either subunit occurring in one of 15 different alternate states.

If this model is correct, there are some further implications. If the subunits of a pair may have identical specificities, then A_{1-1} is incompatible with A_{1-1}, A_{1-2}, A_{2-1} and A_{2-2} for reasons given above. All four *A* factors, when intercrossed, would appear to have the same specificity since they are cross incompatible. If these four *A* factors were present in a population with A_{3-4}, A_{5-6}, A_{7-8}, etc., and if they are equally fertile with the other factors, and the population is large enough, it might be expected that all four would be retained. If, however, the population contains other factors with identical subunits, then all such factors will be at a selective disadvantage compared with the others since they are unable to mate with each other and can mate with fewer nonidentical pairs than the nonidentical pairs themselves. Thus, A_{1-2} or A_{2-1} could mate with A_{9-9} while A_{1-1} and A_{2-2} could not; also A_{1-2} can mate with A_{1-3} but A_{1-1} cannot.

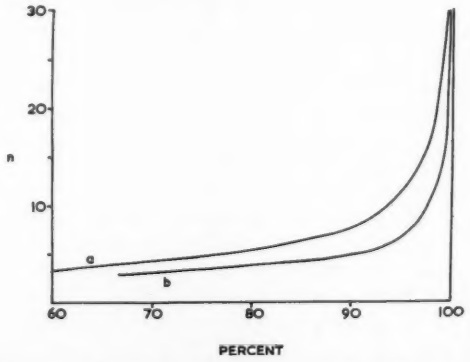


FIGURE 2.—The percentage (P) of random matings, which are fertile, plotted against n, the number of specificities at the two subunits of the *A* mating type locus. Curve a, including identical pairs, $P=\frac{n^3-7n+6}{n^3}\times 100$.

Curve b, excluding identical pairs, $P=\frac{(n+1)(n-2)}{n(n-1)}\times 100$.

These considerations imply that identical subunits will almost certainly be rare in nature and may, in fact, only be found in the laboratory—through recognizing that two cross incompatible factors, A_{1-2} and A_{2-1}, may have different structures.

Curve b in Figure 2 shows that the frequency of cross compatible matings is increased by removing *A* factors with identical subunits. A minimum of nine different subunits are needed to account for RAPER's finding that 97.05 percent of random matings between *A* factors were fertile. RAPER has already identified nine specificities at one *A* subunit and 19 at the other in his collections of Schizophyllum (J. R. RAPER, personal communication).

PAPAZIAN (1958) and RAPER *et al.* (1958b) have both speculated on models for the structure of the *A* locus and have arrived at estimates of the numbers of specificities at each subunit to account for the numbers of *A* and *B* alleles found in nature. In both Schizophyllum and Coprinus the evidence so far indicates only two subunits. No clear example of recombination within subunits has been found. From our current knowledge of the structure of the gene (PONTECORVO 1959), it seems most likely that the subunits are made up of smaller recombination units. It is also very likely that crossing over within the subunits would lead to nonparental subunit specificities detectable by the mating reaction. To obtain 32 different subunit specificities would require only five different sites each with two alternate states. The five percent upper fiducial limit on the total length of the two subunits was calculated from the data in Table 4 as the recombination fraction 2.08×10^{-5}. Recombination of this frequency or greater would have been detected unless both pairs of subunits in the cross $A_5 \times A_6$ and, hence, $A_x \times A_y$ were heterozygous at only one site. Since this is unlikely, we may conclude that the sites within the subunits are very tightly linked.

SUMMARY

(1) Crosses between monocaryotic stocks of *Coprinus lagopus* bearing markers linked with the *A* mating type locus yield a low frequency of nonparental *A* factors in tetrads and random spores. The marker arrangements in these nonparental cultures show that the new *A* factors arise as a result of crossing over between subunits of the *A* locus. Less extensive tests of the *B* locus have not revealed nonparental recombinants.

(2) Seventy-nine nonparental *A* factors, isolated with the aid of linked selective markers, from crosses between A_5 and A_6 were of two types only, suggesting that only two subunits were able to recombine and give nonparental *A* factors. The original parental factor A_5 was recovered by intercrossing the two recombinant factors. The frequency of recombination between the subunits was 0.068 to 0.088 percent.

(3) It is suggested that the two subunits of the *A* locus arose by duplication of a pre-existing unit. The possible structure of the subunits and the gentical basis of their specificity are briefly considered.

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LITERATURE CITED

ANDERSON, G. E., 1959 Induced mutants in *Coprinus lagopus*. (Abstr.) Heredity **13**: 411–412.

BREWBAKER, J. L., and N. SHAPIRO, 1959 Homozygosity and *S* gene mutation. Nature **183**: 1209–1210.

DAY, P. R., 1959a A cytoplasmically controlled abnormality of the tetrads of *Coprinus lagopus*. Heredity **13**: 81–87.

1959b 49th Ann. Rept. John Innes Horticultural Institution for 1958. pp. 17–18.

DAY, P. R., and R. HOLLIDAY, 1959 Incompatibility in *Schizophyllum commune*. Am. Naturalist **93**: 387–388.

KNIEP, H., 1930 Über Selektionswirkungen in fortlaufenden Massenaussaaten von Schizophyllum. Z. Botan. **23**: 510–536.

LEUPOLD, U., 1958 Studies on recombination in *Schizosaccharomyces pombe*. Cold Spring Harbor Symposia Quant. Biol. **13**: 161–170.

LEWIS, D., and L. K. CROWE, 1953 Theory of revertible mutations. Nature **172**: 501.

PANDEY, K. K., 1956 Mutations of self-incompatibility alleles in *Trifolium pratense* and *T. repens*. Genetics **41**: 327–343.

PAPAZIAN, H. P., 1951 The incompatibility factors and a related gene in *Schizophyllum commune*. Genetics **36**: 441–459.

1958 The genetics of basidiomycetes. Advances in Genet. **9**: 41–69.

PITTENGER, T. H., 1954 The general incidence of pseudo-wild types in *Neurospora crassa*. Genetics **39**: 326–342.

PONTECORVO, G., 1959 *Trends in Genetic Analysis*. University Press. Oxford.

RAPER, J. R., G. S. KRONGELB, and M. G. BAXTER, 1958a The number and distribution of incompatibility factors in Schizophyllum. Am. Naturalist **92**: 221–232.

RAPER, J. R., M. G. BAXTER, and R. B. MIDDLETON, 1958b The genetic structure of the incompatibility factors in *Schizophyllum commune*. Proc. Natl. Acad. Sci. U.S. **44**: 889–900.

SWIEZYNSKI, K. M., and P. R. DAY, 1960 Heterocaryon formation in *Coprinus lagopus*. Genetical Res. **1**: 60–74.

TAKEMARU, T., 1957 Genetics of *Collybia velutipes*. V. Mating patterns between F_1-mycelia of legitimate and illegitimate origins in the strain NL-55. Botan. Mag. (Tokyo) **70**: 244–249.

TERAKAWA, H., 1957 The nuclear behaviour and morphogenesis in *Pleurotus ostreatus*. Scientific Papers of the College of Gen. Educ. Univ. Tokyo **7**: 61–88.

WHITEHOUSE, H. L. K., 1949 Multiple-allelomorph heterothallism in the fungi. New Phytologist **48**: 212–244.

A STUDY OF PACHYTENE CHROMOSOME PAIRING IN A CORN-TRIPSACUM HYBRID DERIVATIVE[1]

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THE concept of chromosome homology is not a precise one. When pressed for a definition, we may say that cytologically, homologous chromosome regions are those which seem by and large to match and are found synapsed at pachytene, but we must immediately amend this by saying that under some conditions non-homologous chromosome regions are also found closely paired at pachytene (McCLINTOCK 1933). These latter regions are considered non-homologous because we know that pairing partners which match each more closely exist elsewhere. The pairing of these non-homologous regions is thought to be a haphazard satisfaction of a generalized tendency of single parts to pair, but may be indistinguishable, even when chromomere patterns are compared, from the pairing of homologous regions. It is also true that under certain conditions chromosome regions which are homologous are sometimes found not to be paired at pachytene. It is not clear whether such pachytene pairing failure is due to synaptic failure or to precocious opening of synapsed chromosomes, but it is found in a variety of plants each homozygous for a type of recessive gene usually called "asynaptic" and, perhaps generally, in slightly unbalanced complements (MORGAN 1956; MAGUIRE 1960). Thus pachytene pairing of homologous chromosome regions is subject to genetic and environmental interference. Crossing over occurs regularly at meiosis (with an impressive list of exceptions) between homologous chromosome regions.

It is implicit in cytogenetic theory that homologous chromosome regions carry identical loci. At any locus several allelic forms may be possible, but chromosome pairs which differ only in that they are heterozygous at one or more of their loci are considered homologous throughout their length. Since pachytene pairing appears to be locally specific in structural hybrids, the extrapolation is commonly made that homologous genes are the specific units of pairing affinity, with the gross chromosome pairing observed as a composite effect of many small parts. It seems probable that if specific pairing affinities exist on the level of individual genes, that these affinities vary in strength from one locus to another and between different alleles at a given locus, as well as with factors external to these genes. To the writer's knowledge no direct evidence is available on the question of whether heterozygous genes have as strong an affinity for each other as homozygous ones, although this question might be amenable to experimentation. It should be borne

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in mind that the term "heterozygous" covers gene pairs which may differ only slightly in effect, and presumably in constitution, and also gene pairs which differ widely in effect, and differ to an unknown, but probably (some of the time at least) to a much greater extent, in constitution. Such genes are called alleles because they seem to occupy the same position. Initially identical loci theoretically may come to differ in divergent groups with the accumulation of differences in interaction, or with changes in details of chromosome structure, until their common origin is no longer genetically detectable. During the divergent process similar chromosome regions seem to lose their facility for crossing over and their specific pairing affinity. Synapsis is sometimes imperfect (although no structural differences between the two chromosome sets are apparent) in hybrids between different strains of a species as well as in interspecific hybrids.

Thus, although the generalization is convenient that homologous chromosomes are paired at pachytene and carry the same kinds of genes in the same order, we may expect in practice to find many cases of similar chromosome regions which cannot be so neatly categorized. In separate species a locus may have the same alleles, alleles which differ only in degree of effect, or alleles which differ more widely in effect (FABERGÉ 1944). As shown in the present paper and elsewhere, in interspecific hybrids and their progeny chromosomes or chromosome segments may synapse regularly, or regularly only when a closer homologue is not available, or only so often as segments considered non-homologous usually pair. This paper describes work on corn backcross progenies of corn-Tripsacum hybrids. References to chromosome homology are made with the realization that the concept is a relative one

MATERIALS AND METHODS

The plant material studied was a 21 chromosome stock containing a pair of Zea-Tripsacum interchange chromosomes as illustrated in Figure 1. The interchange occurred in an early corn backcross generation of a Zea-Tripsacum hybrid and involved a distal portion of the short arm of corn chromosome 2 and a distal portion of the long arm of a Tripsacum chromosome which was itself about half as long as corn chromosome 2. The segment from the Tripsacum chromosome, now located in the short arm of chromosome 2, carried a terminal knob and at least two loci normally present in the region of corn chromosome 2 for which it had been exchanged. The segment of the Tripsacum chromosome containing its centromere, now attached to the distal segment from corn chromosome 2, showed no apparent homology to any corn chromosome region.

In 20 chromosome plants heterozygous for the exchange, the interchange chromosome 2 was usually found paired at pachytene with the normal corn chromosome 2 in a perfectly regular fashion so that, except for the presence of the terminal knob, the bivalent could not be distinguished from one comprised of two normal corn chromosomes 2. Analyses of occasional pairing failures, as well as genetic recombination, which occurs only very rarely in this region, have been set forth elsewhere (MAGUIRE 1960).

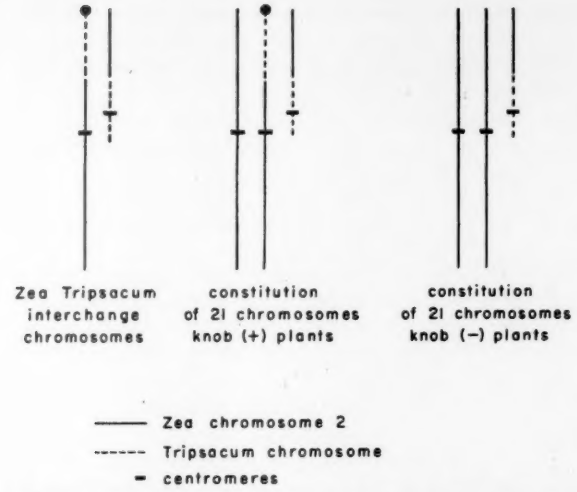

Zea Tripsacum interchange chromosomes
constitution of 21 chromosomes knob (+) plants
constitution of 21 chromosomes knob (−) plants
Zea chromosome 2
Tripsacum chromosome
centromeres

FIGURE 1.—Diagrams showing composition of Zea-Tripsacum interchange chromosomes and the combinations in which they occur in knob (+) and knob (−) plants.

Twenty-one chromosome plants were of two types, those carrying both interchange chromosomes, which will be called knob (+) plants, and those carrying two normal corn chromosomes 2 and the interchange chromosome containing the apparently non-homologous portion of the Tripsacum chromosome attached to the distal segment of the short arm of corn chromosome 2 (Figure 1). The latter type, which will be called knob (−), contained this distal segment of the short arm of corn chromosome 2 in triplicate. Metaphase trivalent frequencies have been found usually to approximate 90 percent in knob (+) plants and 69 percent in knob (−) plants (MAGUIRE 1957). Trivalent configurations at pachytene were of the types illustrated in Figures 2 and 3. All plants were the progeny of seven 21 chromosome knob (+) plants pollinated by a chromosome 2 genetic tester stock.

This study was undertaken to analyze more fully the pairing behavior of the chromosomes described above and to estimate the physical length of the Tripsacum segment exchanged for a portion of corn chromosome 2 of equal length. This length is expected to be equal to that length of the interchange chromosome 2 which is not paired in trivalent configurations of knob (+) plants, and to the length of normal corn chromosome 2 which synapses with the extra chromosome in these configurations. In the analysis which follows the latter measurement was used for simplicity and to facilitate comparisons to pairing behavior in knob (−) plants. Inference from such measurements is complicated by the fact that in most trivalents all three chromosomes are found to be unpaired in a region (usually short) around their point of branching (the "b" region of Figure 3).

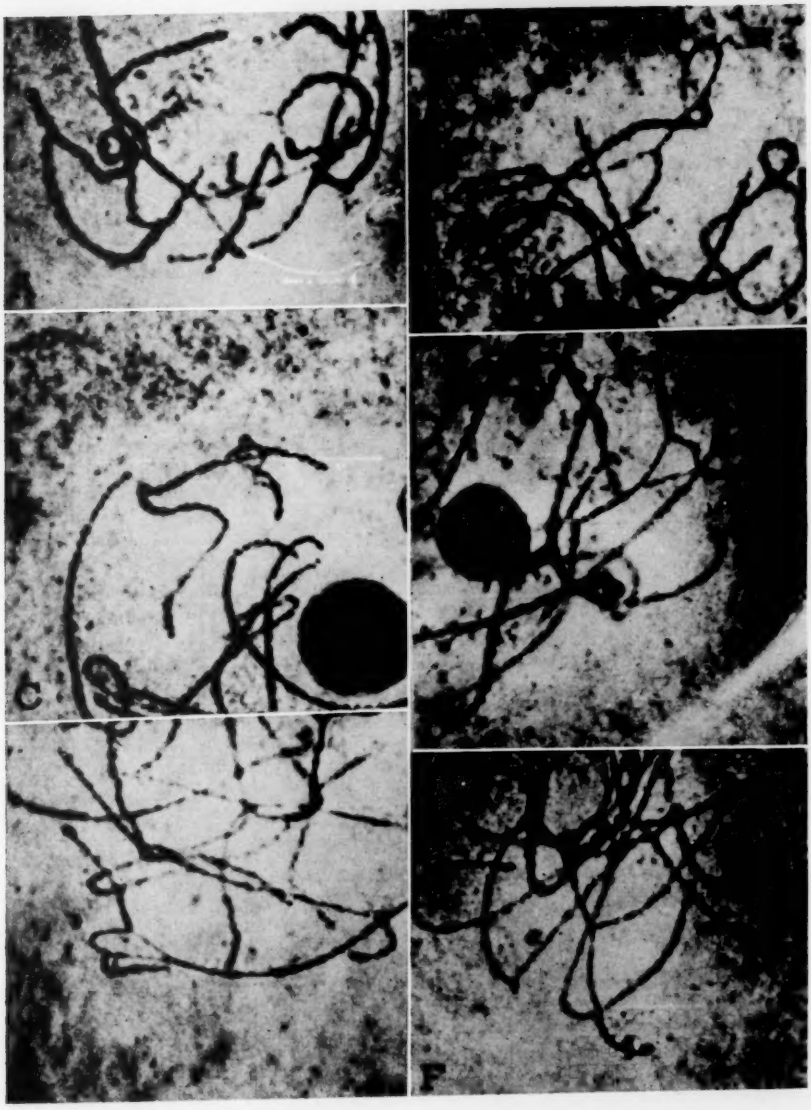
Microsporocyte samples were fixed immediately after collection in alcohol-acetic 3:1 mixture and refrigerated until microscopically examined in aceto-carmine smears. Camera lucida drawings were made (with uniform settings) of all analyzable trivalent configurations from all pachytene cells where chromosomes appeared flat and unstretched. All or nearly all available pachytene material was examined. Some bias may have been imposed on the data by the necessary selection for study of analyzable cells.

RESULTS

The mean of 170 measurements of the length of the extra chromosome at pachytene when involved in a trivalent configuration was 44.1 percent of the total length of corn chromosome 1. The fact that this figure did not differ significantly from the mean of 54 measurements of the extra chromosome as a univalent (44.3 percent of the total length of chromosome 1) supports the validity of comparisons between linear measurements of paired and unpaired chromosome regions.

A total of 115 measurements in 16 knob (+) plants and 54 measurements of five knob (−) plants were made of the percent of the total length of chromosome 2 which was found to be paired in trivalents with the extra chromosome (the "a" region of Figure 3). F tests of whether variances differed among samples from different knob (+) plants or among samples from different knob (−) plants indicated that they did not at the five percent level, and analysis of variance indicated that plant sample means did not differ among knob (+) plants or among knob (−) plants at the five percent level. Therefore, all data were pooled for knob (+) plants and compared to data pooled from knob (−) plants. The cumulative percent frequency distributions of this measurement in knob (+) and knob (−) plants (Figure 4) were tested for goodness of fit to normal distributions by chi-square tests with class intervals set so as to contain equal expected frequencies as suggested by TATE and CLELLAND (1957). The sample distributions were

FIGURE 2.—Photomicrographs of trivalent configurations. A.—Trivalent from a knob (+) plant with the segment of corn chromosome 2 (located in the extra chromosome) paired with the unaltered corn chromosome 2. The knob-bearing segment from the Tripsacum chromosome is unpaired. There is pairing failure in the region of branching of the trivalent. B.—Trivalent from a knob (−) plant with the distal region of the short arm of corn chromosome 2 (located in the extra chromosome) paired with one of the unaltered chromosomes 2. There is pairing failure in the region of branching of the trivalent. C.—Trivalent from a knob (−) plant with the extra chromosome paired with a corn chromosome 2 as in A and B above. In addition there is non-homologous pairing of the Tripsacum segment of the extra chromosome with the end of the other corn chromosome 2 for a short distance. There is pairing failure in the region of branching of the trivalent. D.–E.—Trivalents from knob (+) plants with the corn segment in the extra chromosome paired with the unaltered corn chromosome 2 as in A, B, and C above. In addition there is non-homologous pairing between the two Tripsacum segments. There is pairing failure in the region of branching of the trivalent. F.—Same as D and E but with pairing complete except for a short region near the terminal knob. All types of configuration occurred in all plants. Magnification is about 1000 ×.


C
F

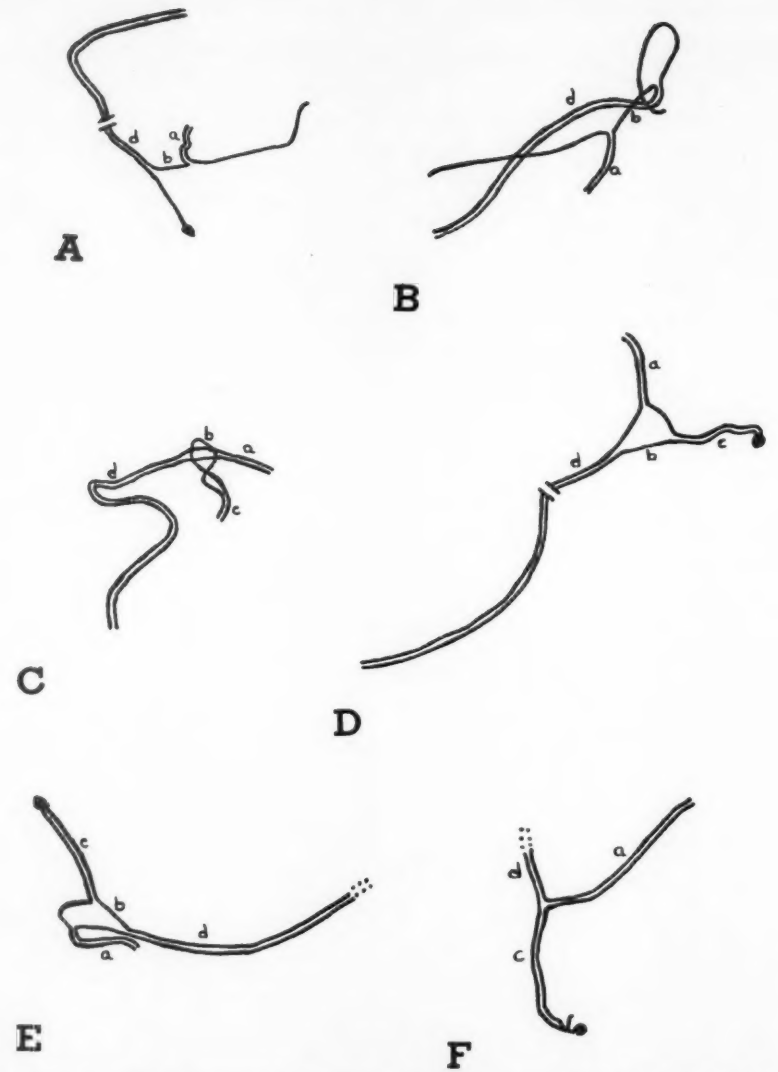


FIGURE 3.—Semidiagrammatic interpretations of the photomicrographs of Figure 2. Various regions are labelled as follows: a.—pairing of distal segments of corn chromosome 2, one located in an unaltered chromosome, the other attached to a Tripsacum segment in the extra chromosome. b.—region of pairing failure. c.—non-homologous pairing: of two Tripsacum segments in trivalents of knob (+) plants, or of the Tripsacum segment in the extra chromosome with corn chromosome 2 in trivalents of knob (—) plants. d.—pairing of unaltered portions of corn chromosome 2 in trivalents. Centromeres, where recognizable, are indicated by crossbars.

found not to differ from normal distributions at the five percent level (or even at the ten percent level). In an F test the variance of all measurements from knob (+) plants (70) did not differ significantly from the variance of all measurements of knob (−) plants (68) at the ten percent level. The means of these measurements, however, were found to differ at the one percent level in a t test. The mean for knob (+) plants was 20.2 percent with a five percent confidence interval estimate of 18.9 percent to 21.5 percent, and for knob (−) plants the mean was 14.6 percent with a five percent confidence interval of 12.7 percent to 16.5 percent.

In most trivalents in the region of branching all three chromosomes were found

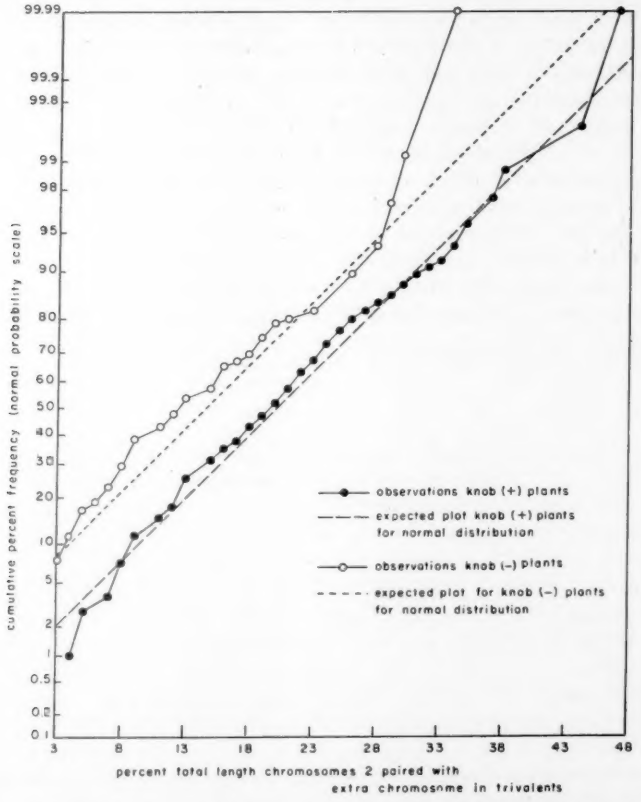


FIGURE 4.—Cumulative percent frequency plots on normal probability scale of observed distributions of the percent total length of chromosome 2 paired with the extra chromosome in trivalent configurations of knob (+) and knob (−) plants. Plots expected for normal distributions with the means and variances observed are also shown.

to be unpaired for a short distance (the "b" region of Figure 3). In the 115 trivalents from knob (+) plants the average of this distance was 8.5 percent of the total length of chromosome 2 with the median at seven percent, and in the 54 trivalents from knob (−) plants the average of this distance was 8.2 percent of the total length of chromosome 2 with a median again at seven percent. A chi-square test of the goodness of fit of these cumulative percent frequency distributions (with class intervals again set so as to contain equal expected frequencies) indicated that they differed significantly from normal distributions at the one percent level. These cumulative frequency distributions of knob (+) and (−) plants were, however, very similar as illustrated in Figure 5. They were characterized by larger frequencies at smaller extents. By a KOLMOGOROV-SMIRNOV test the knob (+) and knob (−) samples did not differ significantly at the 20 percent level. Since knob (+) and knob (−) plants did not seem to differ, it appears that the extent of this region of pairing failure was probably not related to the presence of the knob-bearing Tripsacum segment. A rank difference test of correlation of the average length in microns of chromosome 2 in each plant to the average percent of its length unpaired gave an rd (rank difference correlation coefficient) of −0.57 which is significant at the one percent level (Figure 6). Such absolute measurements of total length are probably influenced by the amount of pressure applied in making the squash preparations, but in a general way presumably reflect the relative stages of advancement of the cells, with more advanced cells showing shorter lengths. It appears then, that this pairing failure increases somewhat with stage advancement and may represent an opening, probably in both directions, after initial pairing, rather than a failure to synapse.

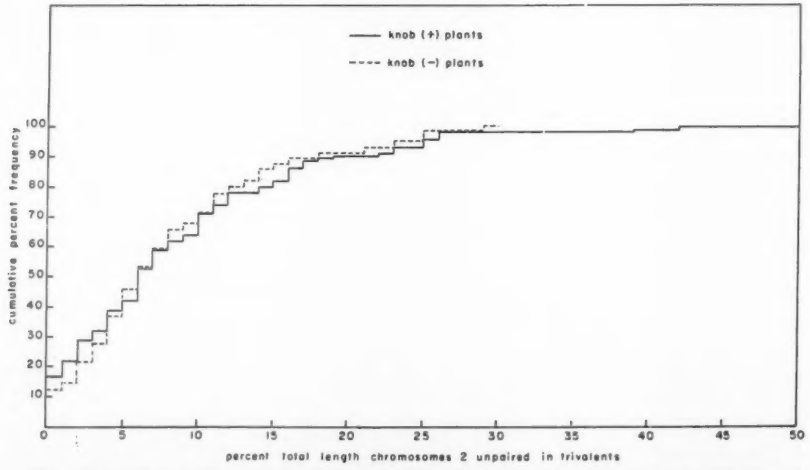


FIGURE 5.—Cumulative percent frequency polygons of the percent of the total length of chromosome 2 found to be unpaired in trivalent configurations from knob (+) and knob (−) plants.

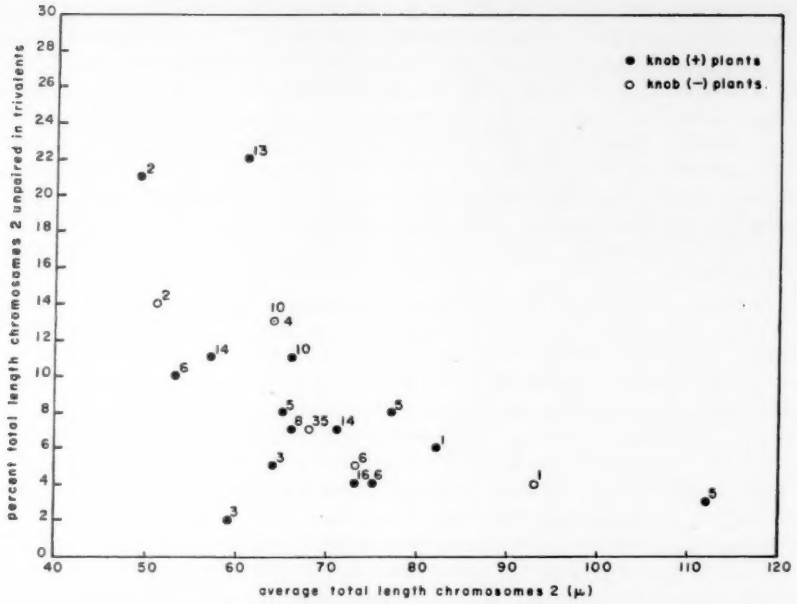


FIGURE 6.—Percent total length chromosome 2 found to be unpaired in trivalent configurations as a function of the average total length of chromosome 2. Each point represents the average values for a plant, and the number beside it is the number of observations which were averaged to give this value.

The average extent of this region of pairing failure must be considered in the estimation of the length of the exchanged chromosome segments. If it is assumed that an opening occurs and proceeds at an equal rate in both directions, an estimation of the length of chromosome 2 which has been replaced by the Tripsacum segment is given by adding half the average extent of percent of chromosome 2 where pairing failure occurred (about 4.2 percent), to the average percent of chromosome 2 found paired with the extra chromosome (20.2 percent) in trivalents of knob (+) plants. This total is 24.4 percent of the total length of chromosome 2. In 19 of the 115 trivalents of knob (+) plants there was virtually no pairing failure. The average extent of pairing of the extra chromosome with the normal chromosome 2 in these trivalents was about 26 percent. This may be considered a direct estimation of the length of segments exchanged, and although it is based on a small number of measurements, it is in fair agreement with the estimate made above. It seems probable that the extent of chromosome 2 replaced by a similar segment from Tripsacum (of the same length) was about 24 to 25 percent of its total length. It cannot be concluded, however, whether or not the Tripsacum chromosome from which the knob-bearing segment was derived con-

tained this region as a segment from previous corn admixture. So far there has been no adequate test of whether the remainder of the Tripsacum chromosome also bears partial homology to part (or parts) of corn chromosome 2. Since early backcross generations where pachytene analysis was not possible, this region has been present only in 21 chromosome plants where it is attached to the distal segment from the short arm of corn chromosome 2. Under these conditions preferential pairing of the sort which discriminates against the pairing of the knob-bearing segment with the corresponding segment of corn chromosome 2 in trivalents, would also prevent the pairing of this region. The exchange which produced the interchange chromosomes described here may have been a crossover type of event.

As indicated in Figures 2 and 3, pairing occurred in some trivalents of knob (+) plants in such a way that the segment of the Tripsacum chromosome transferred to corn chromosome 2 appeared synapsed with the remainder of the Tripsacum chromosome (the "c" region of Figure 3 D, E and F). Unless the chromosome derived from Tripsacum prior to exchange with corn chromosome 2 was an isochromosome with two like regions arranged in reverse order, this pairing is probably non-homologous. Since the centromere of this chromosome was not centrally located (the chromosome's arm ratio was about 3.3:1), it seems unlikely that it was an isochromosome. As shown in Figure 2 and 3, pairing also occurred in trivalents of knob (−) plants between a normal corn chromosome 2 and the Tripsacum segment of the extra chromosome (the "c" region of Figure 3 C). This pairing is also probably non-homologous. The pairing of these regions in knob (+) and knob (−) plants was comparatively irregular. It occurred in 169 of 469 knob (+) sporocytes (about 36 percent) and in 15 of 89 knob (−) sporocytes (about 17 percent). In both cases samples of frequency of occurrence from different plants ranged from zero percent to over 70 percent. Since the difference between knob (+) and knob (−) sporocytes was significant at the one percent level by a chi-square test, there seems to have been a stronger affinity (when there was no other chance for pairing) between two Tripsacum segments than between a corn and a Tripsacum segment, although all these regions were probably non-homologous in the usual sense. Since the variabilities within both knob (+) and knob (−) samples were great, other factors must have been important in determining whether or not this non-homologous pairing occurred.

The extra chromosome was found folded back and paired with itself near its center in seven of the 54 univalents found (about 13 percent). This pairing is similar to the non-homologous pairing which occurred in trivalents of knob (−) plants described above (the "c" region of Figure 3 C). It involves pairing of the centromere carrying Tripsacum segment and distal region from corn chromosome 2.

As reported earlier (MAGUIRE 1957), knob (+) plants usually show a metaphase trivalent frequency of about 90 percent, while knob (−) plants usually show a trivalent frequency of about 69 percent. Heretofore, although almost all metaphase cells could be classified easily as to whether the extra chromosome was involved in a trivalent configuration, only small proportions of pachytene cells

have been classifiable, and there was doubt whether the differences regularly noted between knob (+) and knob (−) plants in metaphase trivalent frequency were due to differences in frequency of trivalent formation at synapsis or to differences in frequency of chiasma formation (MAGUIRE 1957). Some information is now available on this question. In a knob (−) plant it was possible to classify 205 pachytene cells of a total of 563; of these 133, or 65 percent, contained a trivalent. Sixty-one percent of metaphase I cells from this plant showed a trivalent configuration. In a knob (+) plant it was possible to classify 60 pachytene cells of a total of 135; 59 of them or 98 percent showed a trivalent, while 98 percent of metaphase I cells showed a trivalent. This evidence supports the view that chiasma formation usually follows synapsis and that metaphase trivalent frequency gives a fair estimate of trivalent formation at synapsis.

DISCUSSION

It is of interest that the average percent of normal chromosome 2 paired with the extra chromosome in knob (−) plants was significantly less than that in knob (+) plants. The two normal chromosomes 2 in knob (−) plants probably synapse in the region present only in duplicate (the "d" region of Figure 2) in such a way that their pairing on the average extends into the region where three homologous segments are present, before the extra chromosome can pair with one of them for the entire region for which it, too, is homologous. It seems unlikely that the extra chromosome of knob (−) plants consistently differed from that of knob (+) plants since all were siblings of 21 chromosome knob (+) female parents. The normal corn chromosomes 2 of both knob (+) and knob (−) plants were derived from the same genetic tester pollen parents, and it is unlikely that they were structurally altered in knob (−) plants. The behavior could be explained by assuming that pairing is initiated between the two normal corn chromosomes of knob (−) plants somewhere near the usual branching point of trivalents (and perhaps at a number of other points also) at about the same time one of them pairs with the extra chromosome more distally, and then proceeds in both directions at about the same rate. It is also possible that pairing is usually initiated earlier in the region where only two homologs are present, or that it proceeds faster here. It is impossible to choose among the possibilities with the information available. Since trivalents in these knob (−) plants are formed only with a frequency of about 69 percent, about one third of the time the extra chromosome is completely left out as a univalent. This would be expected if a zipper-like closing occurs once contact has been established, and the assumption is made that odds are equal that any two of the three chromosomes will establish contact in the region where all three are homologous.

It is also of interest that the variances of the percent of normal chromosome 2 paired with the extra chromosome in trivalents did not differ significantly at the five percent level in knob (+) and knob (−) samples. A large and similar part of the variation in the two samples was caused by variation in the extent of pairing failure in the region of branching of trivalents. The residual variation must also

be supposed to be similar in knob (+) and knob (−) plants. In knob (+) plants this residual variation would probably be due to differential shortening of various chromosome regions (real and/or artifact of measurement) and conceivably to occasional partial pairing of the corresponding corn and Tripsacum segments. Differential shortening, if it occurs, would probably be equally present in knob (−) plants. Whatever the factors are which influence the extent of pairing with the extra chromosome in knob (−) plants, this distance seems to be about as rigidly determined here as in knob (+) plants where preferential pairing presumably sets this length. Since three homologous segments were present in knob (−) plants, a continuous series of configurations would be expected ranging from trivalents with about 75 to 76 percent of the two normal chromosomes 2 paired with each other, up to nearly 100 percent of these paired. The results do not conform to this expectation but are consistent rather with the interpretation that synapsis is initiated at rather precise positions, or that its course is somehow directed toward a predetermined end no matter how it begins. It is not unreasonable to suppose that different regions of a chromosome may differ in their affinity for the homologue, and that those parts with the strongest tendency to pair would usually do so first. Such regions might or might not be equivalent to the "conjunctive segments" postulated by COOPER (1944) to describe chromosome configurations in meiotic prophase, and metaphase of Olfersia.

A satisfactory explanation of the mechanism of synapsis must take into account preferential pairing of the sort demonstrated by the material described here. In plants where closest homologues are corn and Tripsacum segments, these have been found to pair with a frequency of about 93 percent (MAGUIRE 1960). Two corn segments have been found to pair with a frequency of about 90 percent, however, when the corresponding Tripsacum segment is also available. Two non-homologous Tripsacum segments seem to pair more often (frequency about 36 percent), when no other pairing partner is available, than do corn and Tripsacum non-homologous segments when faced with the same situation (frequency about 17 percent). If pairing is initiated by chance contact of moving chromosomes, as suggested by DELBRUCK (1941), provision must be made for release of relatively non-homologous members (if these should be first to meet) in favor of more closely matched pairs at a later collision. If on the other hand the initiation of synapsis results from specific long range forces of attraction between likes, there must be a hierarchy of degrees of attraction to correspond to the hierarchy of degrees of likeness of chromosome regions. Where three similar parts are present and initially separated in the nucleus, progress toward the meeting of any two could be impeded by the presence of the third.

The mechanical problems of synapsis of greatly extended chromosomes are so great that the suggestion of SMITH (1942a,b), that pairing is generally initiated in the premeiotic anaphase or telophase when chromosomes are more condensed, seems to deserve more consideration than it has received. A number of authors in many groups (reviewed by SMITH) have presented evidence for association of homologues at or by telophase of the last premeiotic division. WILSON (1912) suggested earlier that such premeiotic pairing might occur generally. If a juxta-

position of homologous chromosome regions is achieved at this time, more intimate pairing could be accomplished as the chromosomes uncoil. Such leptotene and zygotene configurations as have been figured would not be at variance with this interpretation when it is recognized that incomplete pairing of chromosomes at early meiotic prophase may represent failure of synapsis or its precocious opening (MAGUIRE 1960). Initial pairing of chromosomes in a condensed state would help to explain why interlocked bivalents occur so rarely at pachytene. In the writer's experience of thousands of corn microsporocytes at pachytene, no convincing case of such interlocking has been seen. In Neurospora where karyogamy immediately precedes meiosis, homologous chromosomes come together in a state at least as condensed as at metaphase and proceed to pair more intimately as they lose their condensation (SINGLETON 1953). Initial pairing of heterochromatic portions in Edessa (SCHRADER 1941) might be interpreted as the coming together of the more condensed regions of homologues. Boss (1954) described chromosome pairing in somatic anaphase of newts.

If condensation facilitates efficient movement of homologous chromosomes toward each other, it is not, of course, to be inferred that chromosomes must pair each time they are condensed. It must be supposed that other causally related special conditions pertain at times when such pairing occurs. The intimate pairing of synapsis might occur only when a loss of condensation immediately follows the juxtaposition of condensed chromosome pairs. If synapsis actually occurs earlier than has been generally supposed, it may precede chromosome reduplication. Under these conditions the hypotheses which require that crossing over occurs during reduplication become more plausible.

SUMMARY

1. Pachytene trivalent configurations were analyzed in 21 chromosome plants of two types. In one type two reciprocal interchange chromosomes were present, one comprised of a corn chromosome 2 with a terminal knob-bearing segment from Tripsacum in the distal region of its short arm, the other a Tripsacum chromosome with the distal region of corn chromosome 2 in its long arm. These are called knob (+) plants. In the other type of 21 chromosome plant, two normal corn chromosomes 2 were present, and the extra chromosome was the Tripsacum chromosome described above with distal region of the short arm of chromosome 2 in its long arm. These are called knob (−) plants.

2. The lengths in the interchange chromosomes of segments exchanged were equal and are estimated to be about 24 to 25 percent of the total length of corn chromosome 2.

3. At the point of partner exchange most trivalents contained a region, usually short, where all chromosomes were unpaired. There was a negative correlation (significant at the one percent level) of the extent of this region with the absolute length of corn chromosome 2. This suggests that the trivalents may have been progressively opening in this region as pachytene advanced.

4. The percent of chromosome 2 which was paired with the extra chromosome

in trivalents was significantly greater in knob (+) plants than in knob (−) plants where the distal segment of corn chromosome 2 was present in triplicate. The variances of this measurement in knob (+) and knob (−) plants did not differ significantly.

5. Theoretical implications are discussed.

LITERATURE CITED

Boss, J., 1954 Mitosis in cultures of newt tissues. II. Chromosome pairing in anaphase. Exptl. Cell Research **7**: 225–231.

Cooper, K. W., 1944 Analysis of meiotic pairing in Olfersia and consideration of the reciprocal chiasmata hypothesis of sex chromosome conjunction in male Drosophila. Genetics **29**: 537–568.

Delbruck, M., 1941 A theory of autocatalytic synthesis of polypeptides and its application to the problem of chromosome reproduction. Cold Spring Harbor Symposia Quant. Biol. **9**: 122–126.

Fabergé, A. C., 1944 Genetics of the Scapiflora section of Papaver. III. Interspecific hybrids and genetic homology. J. Genet. **46**: 125–149.

Maguire, Marjorie P., 1957 Cytogenetic studies of Zea hyperploid for a chromosome derived from Tripsacum. Genetics **42**: 473–486.

1960 A study of homology between a terminal portion of Zea chromosome 2 and a segment derived from Tripsacum. Genetics **45**: 195–209.

McClintock, B., 1933 The association of non-homologous parts of chromosomes in the mid-prophase of meiosis in *Zea mays*. Z. Zellforsch. u. mikroskop. Anat. **19**: 191–237.

Morgan, D. T., 1956 Asynapsis and plasmodial microsporocytes in maize following X-irradiation of the pollen. J. Heredity **47**: 269–274.

Schrader, F., 1941 Heteropycnosis and non-homologous association of chromosomes in *Edessa irrorata* (Hemiptera, Heteroptera). J. Morphol. **69**: 587–604.

Singleton, J. R., 1953 Chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa*. Am. J. Botany **40**: 124–144.

Smith, S. G., 1942a Polarization and progression in pairing—pachytene observations in Neodiprion (Hymenoptera). Can. J. Research **20**: 368–379.

1942b Polarization and progression in pairing. II. Premeiotic orientation and the initiation of pairing. Can. J. Research D. **20**: 221–229.

Tate, Merle W., and Richard C. Clelland, 1957 *Nonparametric and Short-cut Statistics*. Interstate Printers and Publishers. Danville, Illinois.

Wilson, Edmund B., 1912 Studies on chromosomes. VIII. Observation on the maturation phenomenon in certain Hemiptera and other forms, with consideration on synapsis and reduction. J. Exptl. Zool. **13**: 345–450.

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